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PROVISIONAL SPECIFICATION

Invention Title: **Manipulation of condensed tannin biosynthesis 2**

The invention is described in the following statement:

MANIPULATION OF CONDENSED TANNIN BIOSYNTHESIS 2

The present invention relates to nucleic acid fragments encoding amino acid sequences for flavonoid biosynthetic enzymes in plants, and the use thereof for the modification of flavonoid biosynthesis in plants, and more specifically the modification of the content of condensed tannins.

Flavonoids constitute a relatively diverse family of aromatic molecules that are derived from phenylalanine and malonyl-coenzyme A (CoA, via the fatty acid pathway). These compounds include six major subgroups that are found in most higher plants: the chalcones, flavones, flavonols, flavandiols, anthocyanins and condensed tannins (or proanthocyanidins). A seventh group, the aurones, is widespread, but not ubiquitous.

Some plant species also synthesize specialised forms of flavonoids, such as the isoflavonoids that are found in legumes and a small number of non-legume plants. Similarly, sorghum, maize and gloxinia are among the few species known to synthesize 3-deoxyanthocyanins (or phlobaphenes in the polymerised form). The stilbenes, which are closely related to flavonoids, are synthesised by another group of unrelated species that includes grape, peanut and pine.

Besides providing pigmentation to flowers, fruits, seeds, and leaves, flavonoids also have key roles in signalling between plants and microbes, in male fertility of some species, in defence as antimicrobial agents and feeding deterrents, and in UV protection.

Flavonoids also have significant activities when ingested by animals, and there is great interest in their potential health benefits, particularly for compounds such as isoflavonoids, which have been linked to anticancer benefits, and stilbenes that are believed to contribute to reduced heart disease. Condensed tannins which are plant polyphenols with protein-precipitating and antioxidant properties are involved in protein binding, metal chelation, anti-oxidation, and UV-light absorption. As a result condensed tannins inhibit viruses, microorganisms, insects, fungal pathogens, and monogastric digestion. Moderate amounts of

tannins improve forage quality by disrupting protein foam and conferring protection from rumen pasture bloat. Bloat is a digestive disorder that occurs on some highly nutritious forage legumes such as alfalfa (*Medicago sativa*) and white clover (*Trifolium repens*). Moderate amounts of tannin can also reduce digestion rates in the rumen and can reduce parasitic load sufficiently to increase the titre of amino acids and small peptides in the small intestine without compromising total digestion.

The major branch pathways of flavonoid biosynthesis start with general phenylpropanoid metabolism and lead to the nine major subgroups: the colourless chalcones, aurones, isoflavonoids, flavones, flavonols, flavandiols, anthocyanins, condensed tannins, and phlobaphene pigments. The enzyme phenylalanine ammonia-lyase (PAL) of the general phenylpropanoid pathway will lead to the production of cinnamic acid. Cinnamate-4-hydroxylase (C4H) will produce p-coumaric acid which will be converted through the action of 4-coumaroyl:CoA-ligase (4CL) to the production of 4-coumaroyl-CoA and malonyl-CoA. The first committed step channelling carbon into the flavonoid biosynthesis pathway is catalysed by chalcone synthase (CHS), which uses malonyl CoA and 4-coumaryl CoA as substrates.

The *Arabidopsis BANYULS* (BAN) gene encodes a dihydroflavonol 4-reductase-like protein that may be an anthocyanine reductase (ACR). The reaction catalysed by BAN is considered to be one possible branching point from the general flavonoid pathway to the condensed tannin biosynthesis.

An alternative pathway to condensed tannins is via leucoanthocyanidine reductase (LAR). LAR utilises the same substrate as the ACR (BAN) but produces a 2,3-trans isomer as compared to the 2,3-cis isomer produced by ACR.

While nucleic acid sequences encoding the key enzymes in the condensed tannins biosynthetic pathway CHS, BAN and LAR have been isolated for certain species of plants, there remains a need for materials useful in modifying flavonoid biosynthesis and more specifically in modifying condensed tannin biosynthesis and therewith in modifying forage quality, for example by disrupting protein foam

and conferring protection from rumen pasture bloat, particularly in forage legumes and grasses, including alfalfa, medics, clovers, ryegrasses and fescues, and for methods for their use.

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In one aspect, the present invention provides substantially purified or isolated nucleic acids or nucleic acid fragments encoding the key enzymes in the condensed tannins biosynthetic pathway CHS, BAN and LAR from a clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species, or functionally active fragments or variants thereof.

The present invention also provides substantially purified or isolated nucleic acids or nucleic acid fragments encoding amino acid sequences for a class of proteins which are related to CHS, BAN and LAR or functionally active fragments or variants thereof. Such proteins are referred to herein as CHS-like, BAN-like and LAR-like, respectively.

The individual or simultaneous enhancement or otherwise manipulation of CHS, BAN and LAR or like gene activities in plants may enhance or otherwise alter flavonoid biosynthesis; may enhance or otherwise alter the plant capacity for protein binding, metal chelation, anti-oxidation, and UV-light absorption; may enhance or reduce or otherwise alter plant pigment production; and may enhance or otherwise alter the amount of condensed tannins contained within forage legumes and grasses, including alfalfa, medics, clovers, ryegrasses and fescues and therewith the capacity to reduce bloating by disrupting protein foam.

Methods for the manipulation of CHS, BAN and LAR or like gene activities in plants, including legumes such as clovers (*Trifolium* species), lucerne (*Medicago sativa*) and grass species such as ryegrasses (*Lolium* species) and fescues (*Festuca* species) may facilitate the production of, for example, forage legumes and forage grasses and other crops with enhanced tolerance to biotic stresses such as viruses, microorganisms, insects and fungal pathogens; altered

pigmentation in flowers; forage legumes with enhanced herbage quality and bloat-safety.

The clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species may be of any suitable type, including white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*), alfalfa (*Medicago sativa*), Italian or annual ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis*) and red fescue (*Festuca rubra*). Preferably the species is a clover or a ryegrass, more preferably white clover (*T. repens*) or perennial ryegrass (*L. perenne*). White clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) are key pasture legumes and grasses, respectively, in temperate climates throughout the world. Perennial ryegrass is also an important turf grass.

The nucleic acid or nucleic acid fragment may be of any suitable type and includes DNA (such as cDNA or genomic DNA) and RNA (such as mRNA) that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases, and combinations thereof.

In a preferred embodiment of this aspect of the invention, the substantially purified or isolated nucleic acid or nucleic acid fragment encoding a CHS or CHS-like protein includes the nucleotide sequences shown in Figures 2, 6, 10 and 14 hereto; (b) complements of the sequences recited in (a); (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally active fragments and variants of the sequences recited in (a), (b) and (c).

In a further preferred embodiment of this aspect of the invention, the substantially purified or isolated nucleic acid or nucleic acid fragment encoding a BAN or BAN-like protein includes the nucleotide sequence shown in Figure 18 hereto; (b) complements of the sequence recited in (a); (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally active fragments and variants of the sequences recited in (a), (b) and (c).

In a still further preferred embodiment of this aspect of the invention, the substantially purified or isolated nucleic acid or nucleic acid fragment encoding a LAR or LAR-like protein includes the nucleotide sequence shown in Figures 21, 23 and 25 hereto; (b) complements of the sequences recited in (a); (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally active fragments and variants of the sequences recited in (a), (b) and (c).

The term "isolated" means that the material is removed from its original environment (e.g. the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid or polypeptide present in a living plant is not isolated, but the same nucleic acid or polypeptide separated from some or all of the coexisting materials in the natural system, is isolated. Such nucleic acids could be part of a vector and/or such nucleic acids could be part of a composition, and still be isolated in that such a vector or composition is not part of its natural environment. An isolated polypeptide could be part of a composition and still be isolated in that such a composition is not part of its natural environment.

The term "purified" means that the nucleic acid or polypeptide is substantially free of other nucleic acids or polypeptides.

By "functionally active" in respect of a nucleic acid it is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of modifying flavonoid biosynthesis in a plant. Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the nucleotides are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least approximately 80% identity to the relevant part of the above mentioned sequence, more preferably at least approximately 90% identity, most preferably at least approximately 95% identity. Such functionally active variants and fragments include, for example, those having nucleic acid changes which result in conservative amino acid substitutions of one or more residues in the corresponding amino acid sequence. Preferably the fragment has a size of at least

30 nucleotides, more preferably at least 45 nucleotides, most preferably at least 60 nucleotides.

By "functionally active" in respect of a polypeptide is meant that the fragment or variant has one or more of the biological properties of the proteins CHS, CHS-like, BAN, BAN-like, LAR and LAR-like, respectively. Additions, deletions, substitutions and derivatizations of one or more of the amino acids are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least approximately 60% identity to the relevant part of the above mentioned sequence, more preferably at least approximately 80% identity, most preferably at least approximately 90% identity. Such functionally active variants and fragments include, for example, those having conservative amino acid substitutions of one or more residues in the corresponding amino acid sequence. Preferably the fragment has a size of at least 10 amino acids, more preferably at least 15 amino acids, most preferably at least 20 amino acids.

The term "construct" as used herein refers to an artificially assembled or isolated nucleic acid molecule which includes the gene of interest. In general a construct may include the gene or genes of interest, a marker gene which in some cases can also be the gene of interest and appropriate regulatory sequences. It should be appreciated that the inclusion of regulatory sequences in a construct is optional, for example, such sequences may not be required in situations where the regulatory sequences of a host cell are to be used. The term construct includes vectors but should not be seen as being limited thereto.

The term "vector" as used herein encompasses both cloning and expression vectors. Vectors are often recombinant molecules containing nucleic acid molecules from several sources.

By "operatively linked" is meant that said regulatory element(s) is capable of causing expression of said nucleic acid(s) or nucleic acid fragment(s) in a plant cell and said terminator(s) is capable of terminating expression of said nucleic acid(s) or nucleic acid fragment(s) in a plant cell. Preferably, said regulatory

element(s) is upstream of said nucleic acid(s) or nucleic acid fragment(s) and said terminator(s) is downstream of said nucleic acid(s) or nucleic acid fragment(s). In a particularly preferred embodiment, each nucleic acid or nucleic acid fragment has one or more upstream promoters and one or more downstream terminators, although expression of more than one nucleic acid or nucleic acid fragment from an upstream regulatory element(s) or termination of more than one nucleic acid or nucleic acid fragment from a downstream terminator(s) is not precluded.

By "an effective amount" it is meant an amount sufficient to result in an identifiable phenotypic trait in said plant, or a plant, plant seed or other plant part derived therefrom. Such amounts can be readily determined by an appropriately skilled person, taking into account the type of plant, the route of administration and other relevant factors. Such a person will readily be able to determine a suitable amount and method of administration. See, for example, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, the entire disclosure of which is incorporated herein by reference.

Genes encoding other CHS or CHS-like, BAN or BAN-like and LAR or LAR-like proteins, either as cDNAs or genomic DNAs, may be isolated directly by using all or a portion of the nucleic acids or nucleic acid fragments of the present invention as hybridisation probes to screen libraries from the desired plant employing the methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the nucleic acid sequences of the present invention may be designed and synthesized by methods known in the art. Moreover, the entire sequences may be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primer DNA labelling, nick translation, or end-labelling techniques, or RNA probes using available *in vitro* transcription systems. In addition, specific primers may be designed and used to amplify a part or all of the sequences of the present invention. The resulting amplification products may be labelled directly during amplification reactions or labelled after amplification reactions, and used as probes to isolate full-length cDNA or genomic fragments under conditions of appropriate stringency.

In addition, short segments of the nucleic acids or nucleic acid fragments of the present invention may be used in protocols to amplify longer nucleic acids or nucleic acid fragments encoding homologous genes from DNA or RNA. For example, polymerase chain reaction may be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the nucleic acid sequences of the present invention, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding plant genes. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, those skilled in the art can follow the RACE protocol [Frohman *et al.* (1988), *Proc. Natl. Acad. Sci. USA* 85:8998, the entire disclosure of which is incorporated herein by reference] to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Using commercially available 3' RACE and 5' RACE systems (BRL), specific 3' or 5' cDNA fragments may be isolated [Ohara *et al.* (1989), *Proc. Natl. Acad. Sci. USA* 86:5673; Loh *et al.* (1989), *Science* 243:217, the entire disclosures of which are incorporated herein by reference]. Products generated by the 3' and 5' RACE procedures may be combined to generate full-length cDNAs.

In a second aspect of the present invention there is provided a substantially purified or isolated polypeptide from a clover, (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species, selected from the group consisting of CHS and CHS-like, BAN and BAN-like, and LAR and LAR-like proteins; and functionally active fragments and variants thereof.

The clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species may be of any suitable type, including white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*), alfalfa (*Medicago sativa*), Italian or annual ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis*) and red fescue (*Festuca rubra*). Preferably the species is a clover or a ryegrass, more preferably white clover (*T. repens*) or perennial ryegrass (*L. perenne*).

In a preferred embodiment of this aspect of the invention, the substantially purified or isolated CHS or CHS-like polypeptide includes an amino acid sequence selected from the group consisting of sequences shown in Figures 3, 7, 11 and 15 hereto, and functionally active fragments and variants thereof.

In a further preferred embodiment of this aspect of the invention, the substantially purified or isolated BAN or BAN-like polypeptide includes an amino acid sequence shown in Figure 19 hereto, and functionally active fragments and variants thereof.

In a still further preferred embodiment of this aspect of the invention, the substantially purified or isolated LAR or LAR-like polypeptide includes an amino acid sequence selected from the group consisting of sequences shown in Figures 22, 24 and 26 hereto, and functionally active fragments and variants thereof.

In a further embodiment of this aspect of the invention, there is provided a polypeptide recombinantly produced from a nucleic acid or nucleic acid fragment according to the present invention. Techniques for recombinantly producing polypeptides are well known to those skilled in the art.

Availability of the nucleotide sequences of the present invention and deduced amino acid sequences facilitates immunological screening of cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides may be used to immunise animals to produce polyclonal or monoclonal antibodies with specificity for peptides and/or proteins including the amino acid sequences. These antibodies may be then used to screen cDNA expression libraries to isolate full-length cDNA clones of interest.

In a still further aspect of the present invention there is provided a construct including one or more nucleic acids or nucleic acid fragments according to the present invention.

In a still further aspect of the present invention there is provided a vector including one or more nucleic acids or nucleic acid fragments according to the present invention.

In a preferred embodiment of this aspect of the invention, the vector may include one or several of the following: one or more regulatory elements such as promoters, one or more nucleic acids or nucleic acid fragments according to the present invention and one or more terminators; said one or more regulatory elements, one or more nucleic acids or nucleic acid fragments and one or more terminators being operatively linked.

In a particularly preferred embodiment of the present invention the vector may contain nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and BAN or BAN-like polypeptides, operatively linked to a regulatory element or regulatory elements, such that both CHS or CHS-like and BAN or BAN-like proteins are expressed.

In another particularly preferred embodiment of the present invention the vector may contain nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and LAR or LAR-like polypeptides, operatively linked to a regulatory element or regulatory elements, such that both CHS or CHS-like and LAR or LAR-like proteins are expressed.

In yet another particularly preferred embodiment of the present invention the vector may contain nucleic acids or nucleic acid fragments encoding both LAR or LAR-like and BAN or BAN-like polypeptides, operatively linked to a regulatory element or regulatory elements, such that both LAR or LAR-like and BAN or BAN-like proteins are expressed.

In an even more preferred embodiment of the present invention the vector may contain nucleic acids or nucleic acid fragments encoding all three of CHS or CHS-like, BAN or BAN-like and LAR or LAR-like, operatively linked to a regulatory element or regulatory elements, such that all three of CHS or CHS-like, BAN or BAN-like and LAR or LAR-like proteins are expressed.

The vector may be of any suitable type and may be viral or non-viral. The vector may be an expression vector. Such vectors include chromosomal, non-chromosomal and synthetic nucleic acid sequences, e.g. derivatives of plant viruses; bacterial plasmids; derivatives of the Ti plasmid from *Agrobacterium tumefaciens*, derivatives of the Ri plasmid from *Agrobacterium rhizogenes*; phage DNA; yeast artificial chromosomes; bacterial artificial chromosomes; binary bacterial artificial chromosomes; vectors derived from combinations of plasmids and phage DNA. However, any other vector may be used as long as it is replicable, integrative or viable in the plant cell.

The regulatory element and terminator may be of any suitable type and may be endogenous to the target plant cell or may be exogenous, provided that they are functional in the target plant cell.

Preferably the regulatory element is a promoter. A variety of promoters which may be employed in the vectors of the present invention are well known to those skilled in the art. Factors influencing the choice of promoter include the desired tissue specificity of the vector, and whether constitutive or inducible expression is desired and the nature of the plant cell to be transformed (e.g. monocotyledon or dicotyledon). Particularly suitable promoters include but are not limited to the constitutive Cauliflower Mosaic Virus 35S (CaMV 35S) promoter and derivatives thereof, the maize Ubiquitin promoter, the rice Actin promoter, and the tissue-specific Arabidopsis small subunit (ASSU) promoter.

A variety of terminators which may be employed in the vectors and constructs of the present invention are also well known to those skilled in the art. The terminator may be from the same gene as the promoter sequence or a different gene. Particularly suitable terminators are polyadenylation signals, such as the CaMV 35S polyA and other terminators from the nopaline synthase (*nos*), the octopine synthase (*ocs*) and the rbcS genes.

The vector, in addition to the regulatory element(s), the nucleic acid(s) or nucleic acid fragment(s) of the present invention and the terminator(s), may include further elements necessary for expression of the nucleic acid(s) or nucleic

acid fragment(s), in different combinations, for example vector backbone, origin of replication (ori), multiple cloning sites, recognition sites for recombination events, spacer sequences, enhancers, introns (such as the maize Ubiquitin *Ubi* intron), antibiotic resistance genes and other selectable marker genes [such as the neomycin phosphotransferase (*npt2*) gene, the hygromycin phosphotransferase (*hph*) gene, the phosphinotricin acetyltransferase (*bar* or *pat*) gene and the gentamycin acetyl transferase (*aacC1*) gene], and reporter genes [such as beta-glucuronidase (GUS) gene (*gusA*) and green fluorescent protein (gfp)]. The vector may also contain a ribosome binding site for translation initiation. The vector may also include appropriate sequences for amplifying expression.

As an alternative to use of a selectable marker gene to provide a phenotypic trait for selection of transformed host cells, the presence of the vector in transformed cells may be determined by other techniques well known in the art, such as PCR (polymerase chain reaction), Southern blot hybridisation analysis, histochemical GUS assays, visual examination including microscopic examination of fluorescence emitted by gfp, northern and Western blot hybridisation analyses.

Those skilled in the art will appreciate that the various components of the vector are operatively linked, so as to result in expression of said nucleic acid(s) or nucleic acid fragment(s). Techniques for operatively linking the components of the vector of the present invention are well known to those skilled in the art. Such techniques include the use of linkers, such as synthetic linkers, for example including one or more restriction enzyme sites.

The constructs and vectors of the present invention may be incorporated into a variety of plants, including monocotyledons (such as grasses from the genera *Lolium*, *Festuca*, *Paspalum*, *Pennisetum*, *Panicum* and other forage and turfgrasses, corn, oat, sugarcane, wheat and barley), dicotyledons (such as *Arabidopsis*, tobacco, clovers, medics, eucalyptus, potato, sugarbeet, canola, soybean, chickpea) and gymnosperms. In a preferred embodiment, the vectors may be used to transform monocotyledons, preferably grass species such as ryegrasses (*Lolium* species) and fescues (*Festuca* species), more preferably perennial ryegrass, including forage- and turf-type cultivars. In an alternate

preferred embodiment, the constructs and vectors may be used to transform dicotyledons, preferably forage legume species such as clovers (*Trifolium* species) and medics (*Medicago* species), more preferably white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*) and alfalfa (*Medicago sativa*). Clovers, alfalfa and medics are key pasture legumes in temperate climates throughout the world.

Techniques for incorporating the constructs and vectors of the present invention into plant cells (for example by transduction, transfection or transformation) are known to those skilled in the art. Such techniques include *Agrobacterium*-mediated introduction, electroporation to tissues, cells and protoplasts, protoplast fusion, injection into reproductive organs, injection into immature embryos and high velocity projectile introduction to cells, tissues, calli, immature and mature embryos. The choice of technique will depend largely on the type of plant to be transformed.

In a further aspect of the present invention there is provided a method of isogenic transformation of a dicotyledonous plant, said method including transforming only one of each pair of cotyledons. This enables the production of pairs of transgenic plant and corresponding untransformed negative control in an otherwise isogenic genetic background for detailed functional assessment of the impact of the transgene on plant phenotype. In a preferred embodiment of this aspect of the invention, the method may include isogenic transformation of a dicotyledonous plant with a construct or vector according to the present invention.

Cells incorporating the constructs and vectors of the present invention may be selected, as described above, and then cultured in an appropriate medium to regenerate transformed plants, using techniques well known in the art. The culture conditions, such as temperature, pH and the like, will be apparent to the person skilled in the art. The resulting plants may be reproduced, either sexually or asexually, using methods well known in the art, to produce successive generations of transformed plants.

In a further aspect of the present invention there is provided a plant cell, plant, plant seed or other plant part, including, e.g. transformed with, one or more constructs, vectors, nucleic acids or nucleic acid fragments of the present invention.

The plant cell, plant, plant seed or other plant part may be from any suitable species, including monocotyledons, dicotyledons and gymnosperms. In a preferred embodiment the plant cell, plant, plant seed or other plant part may be from a monocotyledon, preferably a grass species, more preferably a ryegrass (*Lolium* species) or fescue (*Festuca* species), more preferably perennial ryegrass, including both forage- and turf-type cultivars. In an alternate preferred embodiment the plant cell, plant, plant seed or other plant part may be from a dicotyledon, preferably forage legume species such as clovers (*Trifolium* species) and medics (*Medicago* species), more preferably white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*) and alfalfa (*Medicago sativa*).

The present invention also provides a plant, plant seed or other plant part, or a plant extract derived from a plant cell of the present invention.

The present invention also provides a plant, plant seed or other plant part, or a plant extract derived from a plant of the present invention.

In a further aspect of the present invention there is provided a method of modifying condensed tannin biosynthesis; of modifying flavonoid biosynthesis; of modifying protein binding, metal chelation, anti-oxidation, and UV-light absorption; of modifying plant pigment production; of modifying plant defence to biotic stresses such as viruses, microorganisms, insects, fungal pathogens; of modifying forage quality by disrupting protein foam and conferring protection from rumen pasture bloat, said method including introducing into said plant an effective amount of a nucleic acid or nucleic acid fragment, construct and/or vector according to the present invention.

In a further aspect of the present invention there is provided a method of inhibiting bloat in an animal, said method including providing the animal with a forage plant including a construct, vector, nucleic acid or nucleic acid fragment according to the present invention. The animal is preferably a ruminant, including sheep, goats and cattle. The forage plant including a construct vector, nucleic acid or nucleic acid fragment according to the present invention may form all or part of the feed of the animal. The forage plant preferably expresses CHS or CHS-like proteins, BAN or BAN-like proteins, and/or LAR or LAR-like proteins at higher levels than the equivalent wild-type plant. More preferably, the forage plant expresses both CHS or CHS-like proteins and BAN or BAN-like proteins; both CHS or CHS-like proteins and LAR or LAR-like proteins; or both BAN or BAN-like proteins and LAR or LAR-like proteins; at higher levels than the equivalent wild-type plant. More preferably, the forage plant expresses all three of CHS or CHS-like proteins, BAN or BAN-like proteins, and LAR or LAR-like proteins; at higher levels than the equivalent wild-type plant.

In a further aspect of the present invention there is provided a method for enhancing an animal's growth rate, said method including providing the animal with a forage plant including a construct, vector, nucleic acid or nucleic acid fragment according to the present invention. The animal is preferably a ruminant, including sheep, goats and cattle. The forage plant including a construct, vector, nucleic acid or nucleic acid fragment according to the present invention may form all or part of the feed of the animal. The forage plant preferably expresses CHS or CHS-like proteins, BAN or BAN-like proteins, and/or LAR or LAR-like proteins at higher levels than the equivalent wild-type plant. More preferably, the forage plant expresses both CHS or CHS-like proteins and BAN or BAN-like proteins; both CHS or CHS-like proteins and LAR or LAR-like proteins; or both BAN or BAN-like proteins and LAR or LAR-like proteins; at higher levels than the equivalent wild-type plant. More preferably, the forage plant expresses all three of CHS or CHS-like proteins, BAN or BAN-like proteins, and LAR or LAR-like proteins; at higher levels than the equivalent wild-type plant.

It is estimated that the method of enhancing an animal's growth rate according to this invention should result in an increase in, for example, lamb

growth rate of at least approximately 5%, more preferably at least approximately 10%.

Using the methods and materials of the present invention, condensed tannin biosynthesis, flavonoid biosynthesis, protein binding, metal chelation, anti-oxidation, UV-light absorption, tolerance to biotic stresses such as viruses, microorganisms, insects and fungal pathogens; pigmentation in for example flowers and leaves; herbage quality and bloat-safety; isoflavonoid content leading to health benefits, may be increased or otherwise altered, for example by incorporating additional copies of one or more sense nucleic acids or nucleic acid fragments of the present invention. They may be decreased or otherwise altered, for example by incorporating one or more antisense nucleic acids or nucleic acid fragments of the present invention.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the Figures

Figure 1 shows the plasmid map in pGEM-T Easy of TrCHSa3.

Figure 2 shows the nucleotide sequence of TrCHSa3.

Figure 3 shows the deduced amino acid sequence of TrCHSa3.

Figure 4 shows plasmid maps of sense and antisense constructs of TrCHSa3 in the binary vector pPZP221:35S[2].

Figure 5 shows the plasmid map in pGEM-T Easy of TrCHSc.

Figure 6 shows the nucleotide sequence of TrCHSc.

Figure 7 shows the deduced amino acid sequence of TrCHSc.

Figure 8 shows plasmid maps of sense and antisense constructs of TrCHSc in the binary vector pPZP221:35S^2.

Figure 9 shows the plasmid map in pGEM-T Easy of TrCHSf.

Figure 10 shows the nucleotide sequence of TrCHSf.

Figure 11 shows the deduced amino acid sequence of TrCHSf.

Figure 12 shows plasmid maps of sense and antisense constructs of TrCHSf in the binary vector pPZP221:35S^2.

Figure 13 shows the plasmid map in pGEM-T Easy of TrCHSh.

Figure 14 shows the nucleotide sequence of TrCHSh.

Figure 15 shows the deduced amino acid sequence of TrCHSh.

Figure 16 shows plasmid maps of sense and antisense constructs of TrCHSh in the binary vector pPZP221:35S^2.

Figure 17 shows the plasmid map in pGEM-T Easy of TrBANa.

Figure 18 shows the nucleotide sequence of TrBANa.

Figure 19 shows the deduced amino acid sequence of TrBANa.

Figure 20 shows plasmid maps of sense and antisense constructs TrBANa in the binary vector pPZP221:35S^2.

Figure 21 shows the plasmid map in pGEM-T Easy of TrLARa.

Figure 22 shows the nucleotide sequence of TrLARa.

Figure 23 shows the deduced amino acid sequence of TrLARa.

Figure 24 shows plasmid maps of sense and antisense constructs of TrLARa in the binary vector pPZP221:35S^2.

Figure 25 shows the plasmid map in pGEM-T Easy of TrLARb.

Figure 26 shows the nucleotide sequence of TrLARb.

Figure 27 shows the deduced amino acid sequence of TrLARb.

Figure 28 shows plasmid maps of sense and antisense constructs of TrLARb in the binary vector pPZP221:35S^2.

Figure 29 shows the plasmid map in pGEM-T Easy of TrLARc.

Figure 30 shows the nucleotide sequence of TrLARc.

Figure 31 shows the deduced amino acid sequence of TrLARc.

Figure 32 shows plasmid maps of sense and antisense constructs of TrLARc in the binary vector pPZP221:35S^2.

Figure 33 shows the plasmid map of the binary vector pPZP221:ASSU::TrBAN:35S^2::TrCHS.

EXAMPLE 1

Preparation of cDNA libraries, isolation and sequencing of cDNAs coding for CHS, CHS-like, BAN, BAN-like, LAR and LAR-like proteins from white clover (*Trifolium repens*)

cDNA libraries representing mRNAs from various organs and tissues of white clover (*Trifolium repens*) were prepared. The characteristics of the white clover libraries are described below (Table 1).

TABLE 1

cDNA libraries from white clover (*Trifolium repens*)

Library	Organ/Tissue
01wc	Whole seedling, light grown
02wc	Nodulated root 3, 5, 10, 14, 21 &28 day old seedling
03wc	Nodules pinched off roots of 42 day old rhizobium inoculated plants
04wc	Cut leaf and stem collected after 0, 1, 4, 6 &14 h after cutting
05wc	Inflorescences: <50% open, not fully open and fully open
06wc	Dark grown etiolated
07wc	Inflorescence – very early stages, stem elongation, < 15 petals, 15-20 petals
08wc	seed frozen at –80°C, imbibed in dark overnight at 10°C
09wc	Drought stressed plants
10wc	AMV infected leaf
11wc	WCMV infected leaf
12wc	Phosphorus starved plants
13wc	Vegetative stolon tip
14wc	stolon root initials
15wc	Senescing stolon
16wc	Senescing leaf

The cDNA libraries may be prepared by any of many methods available. For example, total RNA may be isolated using the Trizol method (Gibco-BRL, USA) or the RNeasy Plant Mini kit (Qiagen, Germany), following the manufacturers' instructions. cDNAs may be generated using the SMART PCR cDNA synthesis kit (Clontech, USA), cDNAs may be amplified by long distance polymerase chain reaction using the Advantage 2 PCR Enzyme system (Clontech, USA), cDNAs may be cleaned using the GeneClean spin column (Bio 101, USA), tailed and size fractionated, according to the protocol provided by Clontech. The cDNAs may be introduced into the pGEM-T Easy Vector system 1 (Promega, USA) according to the protocol provided by Promega. The cDNAs in the pGEM-T Easy plasmid vector are transfected into *Escherichia coli* Epicurean coli XL10-

Gold ultra competent cells (Stratagene, USA) according to the protocol provided by Stratagene.

Alternatively, the cDNAs may be introduced into plasmid vectors for first preparing the cDNA libraries in Uni-ZAP XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA, USA). The Uni-ZAP XR libraries are converted into plasmid libraries according to the protocol provided by Stratagene. Upon conversion, cDNA inserts will be contained in the plasmid vector pBlueScript. In addition, the cDNAs may be introduced directly into precut pBlueScript II SK(+) vectors (Stratagene) using T4 DNA ligase (New England Biolabs), followed by transfection into *E. coli* DH10B cells according to the manufacturer's protocol (GIBCO BRL Products).

Once the cDNA inserts are in plasmid vectors, plasmid DNAs are prepared from randomly picked bacterial colonies containing recombinant plasmids, or the insert cDNA sequences are amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Plasmid DNA preparation may be performed robotically using the Qiagen QiaPrep Turbo kit (Qiagen, Germany) according to the protocol provided by Qiagen. Amplified insert DNAs are sequenced in dye-terminator sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"). The resulting ESTs are analysed using an Applied Biosystems ABI 3700 sequence analyser.

EXAMPLE 2

DNA sequence analyses

The cDNA clones encoding CHS, CHS-like, BAN, BAN-like, LAR and LAR-like proteins were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul *et al.* (1993), *J. Mol. Biol.* 215:403-410) searches. The cDNA sequences obtained were analysed for similarity to all publicly available DNA sequences contained in the eBioInformatics nucleotide database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared

for similarity to all publicly available protein sequences contained in the SWISS-PROT protein sequence database using BLASTx algorithm (v 2.0.1) (Gish and States (1993), *Nature Genetics* 3:266-272) provided by the NCBI.

The cDNA sequences obtained and identified were then used to identify additional identical and/or overlapping cDNA sequences generated using the BLASTN algorithm. The identical and/or overlapping sequences were subjected to a multiple alignment using the CLUSTALw algorithm, and to generate a consensus contig sequence derived from this multiple sequence alignment. The consensus contig sequence was then used as a query for a search against the SWISS-PROT protein sequence database using the BLASTx algorithm to confirm the initial identification.

EXAMPLE 3

Identification and full-length sequencing of cDNAs encoding white clover CHS, BAN and LAR proteins

To fully characterise for the purposes of the generation of probes for hybridisation experiments and the generation of transformation vectors, a set of cDNAs encoding white clover CHS, BAN and LAR proteins was identified and fully sequenced.

Full-length cDNAs were identified from our EST sequence database using relevant published sequences (NCBI databank) as queries for BLAST searches. Full-length cDNAs were identified by alignment of the query and hit sequences using Sequencher (Gene Codes Corp., Ann Arbor, MI 48108, USA). The original plasmid was then used to transform chemically competent XL-1 cells (prepared in-house, $CaCl_2$ protocol). After colony PCR (using HotStarTaq, Qiagen) a minimum of three PCR-positive colonies per transformation were picked for initial sequencing with M13F and M13R primers. The resulting sequences were aligned with the original EST sequence using Sequencher to confirm identity and one of the three clones was picked for full-length sequencing, usually the one with the best initial sequencing result.

Sequencing of TrBAN could be completed with M13F and M13R primers. Sequencing of TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrLARa, TrLARb and TrLARc was completed by primer walking, i.e. oligonucleotide primers were designed to the initial sequence and used for further sequencing. The sequences of the oligonucleotide primers are shown in Table 2.

Contigs were then assembled in Sequencher. The contigs include the sequences of the SMART primers used to generate the initial cDNA library as well as pGEM-T Easy vector sequence up to the EcoRI cut site both at the 5' and 3' end.

Plasmid maps and the full cDNA sequences of TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc proteins were obtained (Figures 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21, 22, 25, 26, 29 and 30).

TABLE 2

List of primers used for sequencing of the full-length cDNAs of TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrLARa, TrLARb and TrLARc

gene name	clone ID	sequencing primer	primer sequence (5'>3')
TrCHSa3	05wc1RsB06	05wc1RsB06.f1	AGGAGGCTGCAGTCAAGG
		05wc1RsB06.f2	TGCCTGAAATTGAGAAACC
		05wc1RsB06.f3	AAAGCTAGCCTTGAAGCC
TrCHSc	07wc1TsE12	07wc1TsE12.f1	TCGGACATAACTCATGTGG
		07wc1TsE12.f2	TTGGGTTGGAGAATAAGG
		07wc1TsE12.r1	TGGACATTTATTGGTTGC
		07wc1TsE12.r2	TATCATGTCTGGAAATGC
TrCHSf	07wc1UsD07	07wc1UsD07.f1	AGATTGCATCAAAGAATGG
		07wc1UsD07.r1	GGTCCAAAAGCCAATCC
TrCHSh	13wc2IsG04	13wc2IsG04.f1	TAAGACGAGACATAGTGG
		13wc2IsG04.r1	TATTCACTAAGCACATGC
TrLARa	05wc1CsA02	05wc1CsA02.f1	TCATTTCTGCAATAGGAGG
		05wc1CsA02.r1	ATCCACCTCAGGTGAACC
TrLARb	05wc3EsA03	05wc3EsA03.f1	AATAGGAGGCTCTGATGG

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		05wc3EsA03r1	ATCCACCTCAGGTGAACC
TrLARc	07wc1VsF06	07wc1VsF06.f1	AGGCTCTGATGGCTTGC
		07wc1VsF06.r1	ATCCACCTCAGGTGAACC

EXAMPLE 4

Development of binary transformation vectors containing chimeric genes with cDNA sequences from white clover TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc

To alter the expression of the proteins involved in flavonoid biosynthesis, and more specifically condensed tannin biosynthesis to improve herbage quality and bloat-safety, a set of sense and antisense binary transformation vectors was produced.

cDNA fragments were generated by high fidelity PCR using the original pGEM-T Easy plasmid cDNA as a template. The primers used (Table 3) contained recognition sites for appropriate restriction enzymes, for example EcoRI and XbaI, for directional and non-directional cloning into the target vector. After PCR amplification and restriction digest with the appropriate restriction enzyme (usually XbaI), the cDNA fragments were cloned into the corresponding site in a modified pPZP binary vector (Hajdukiewicz *et al.*, 1994). The pPZP221 vector was modified to contain the $35S^2$ cassette from pKYLX71:$35S^2$ as follows: pKYLX71:$35S^2$ was cut with ClaI. The 5' overhang was filled in using Klenow and the blunt end was A-tailed with Taq polymerase. After cutting with EcoRI, the 2kb fragment with an EcoRI-compatible and a 3'-A tail was gel-purified. pPZP221 was cut with HindIII and the resulting 5' overhang filled in and T-tailed with Taq polymerase. The remainder of the original pPZP221 multi-cloning site was removed by digestion with EcoRI, and the expression cassette cloned into the EcoRI site and the 3' T overhang restoring the HindIII site. This binary vector contains between the left and right border the plant selectable marker gene aacC1 under the control of the 35S promoter and 35S terminator and the pKYLX71:$35S^2$-derived expression cassette with a CaMV 35S promoter with a duplicated enhancer region and an rbcS terminator. Alternatively, the primers contained *att*B sequences for use with

recombinases utilising the GATEWAY® system (Invitrogen). The resulting PCR fragments were used in a recombination reaction with pDONR® vector (Invitrogen). A GATEWAY® cloning cassette (Invitrogen) was introduced into the multicloning site of the pPZP221:35S^2 vector following the manufacturer's protocol. In a further recombination reaction, the cDNAs encoding the open reading frame sequences were transferred from the pDONR® vector to the GATEWAY®-enabled pPZP221:35S^2 vector.

The orientation of the constructs (sense or antisense) was checked by restriction enzyme digest and sequencing which also confirmed the correctness of the sequence. Transformation vectors containing chimeric genes using full-length open reading frame cDNAs encoding white clover TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc proteins in sense and antisense orientation under the control of the CaMV 35S^2 promoter were generated (Figures 4, 8, 12, 16, 20, 24, 28 and 32).

TABLE 3

List of primers used to PCR-amplify the open reading frames

gene name	primer	primer sequence (5'->3')
TrCHSa3	05wc1RsB06f	GAATTCTAGAAGATATGGTGAGTGTAGCTG
	05wc1RsB06r	GAATTCTAGAATCACACATCTTATATAGCC
TrCHSa3	05wc1RsB06fG	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTAGAAGATATGGTGAGTGTAGCTG
	05wc1RsB06rG	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGAATCACACATCTTATATAGCC
TrCHSc	07wc1TsE12f	GAATTCTAGAAGAAGAAATATGGGAGACGAAGG
	07wc1TsE12r	GAATTCTAGAAAGACTTCATGCACACAAGTTCC
TrCHSf	07wc1UsD07f	GAATTCTAGATGATTCATTGTTTGTTTCCATAAC
	07wc1UsD07r	GAATTCTAGAACATATTCATCTTCCTATCAC
TrCHSh	13wc2lsG04f	GAATTCTAGATCCAAATTCTCGTACCTCACC
	13wc2lsG04r	GAATTCTAGATAGTTCACATCTCTCGGCAGG
TrBANa	05wc2XsG02f	GGATCCTCTAGAGCACTAGTGTGTATAAGTTTCTTGG
	05wc2XsG02r	GGATCCTCTAGACCCCCTTAGTCTTAAAATACTCG
TrLARa	05wc1CsA02fG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTAGAAAGCAAAGCAATGGCACC
	05wc1CsA02rG	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGAT

		CCACCTCAGGTGAACC
TrLARb	05wc3EsA03fG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTAGAAAGCAATGGCACCAGCAGC
	05wc3EsA03rG	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGATCCACCTCAGGTGAACC
TrLARc	07wc1VsF06fG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTAGATAAAGCAATGGCACCAGC
	07wc1VsF06rG	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGATCCACCTCAGGTGAACC

The pPZP221:35S^2 binary vector was further modified to contain two expression cassettes within one T-DNA. The expression cassette from the binary vector pWM5 consisting of the ASSU promoter and the tob terminator was PCR-amplified with Pwo DNA polymerase using oligonucleotide primers with the following sequences:

forward primer 5'-CCACCATGTTTGAAATTTATTATGTGTTTTTTTCCG-3';
reverse primer 5'-TAATCCCGGGTAAGGGCAGCCCATACAAATGAAGC-3'.

The PCR product was cut with BstXI and SmaI and cloned directionally into the equally cut pPZP221:35S^2 vector. Additionally, a GATEWAY® cloning cassette (Invitrogen) was introduced into the multicloning site in the 35S^2:rbcS expression cassette following the manufacturer's protocol. TrBANa was cloned into the ASSU:tob expression cassette, TrCHSa3 was amplified with the GATEWAY-compatible primers (see Table 3) and cloned into the 35S2:rbcS expression cassette. A transformation vector containing chimeric genes using full-length open reading frame cDNAs encoding white clover TrBANa protein in sense orientation under the control of the ASSU promoter and TrCHSc3 protein in sense orientation under the control of the CaMV 35S^2 promoter within the same T-DNA was generated (Figure 33).

EXAMPLE 6

Production by isogenic transformation and analysis of transgenic white clover plants carrying chimeric white clover TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc genes involved in flavonoid biosynthesis

A set of transgenic white clover plants carrying chimeric white clover genes involved in flavonoid biosynthesis, and more specifically condensed tannin biosynthesis to improve herbage quality and bloat-safety, were produced as detailed in Example 5.

Agrobacterium-mediated gene transfer experiments were performed using these transformation vectors.

The production of transgenic white clover plants carrying the white clover TrCHSa3, TrCHSc, TrCHSf, TrCHSh, TrBANa, TrLARa, TrLARb and TrLARc cDNAs, either singly or in combination, is described here in detail.

Preparation of *Agrobacterium*

Agrobacterium tumefaciens strain AGL-1 transformed with one of the binary vector constructs detailed in Example 6 were streaked on LB medium containing 50 µg/ml rifampicin and 50 µg/ml kanamycin and grown at 27 °C for 48 hours. A single colony was used to inoculate 5 ml of LB medium containing 50 µg/ml rifampicin and 50 µg/ml kanamycin and grown over night at 27 °C and 250 rpm on an orbital shaker. The overnight culture was used as an inoculum for 500 ml of LB medium containing 50 µg/ml kanamycin only. Incubation was over night at 27 °C and 250 rpm on an orbital shaker in a 2 l Erlenmeyer flask.

Preparation of white clover seeds

1 spoon of seeds (ca. 500) was placed into a 280 µm mesh size sieve and washed for 5 min under running tap water, taking care not to wash seeds out of sieve. In a laminar flow hood, seeds were transferred with the spoon into an autoclaved 100 ml plastic culture vessel. A magnetic stirrer (wiped with 70%

EtOH) and ca. 30 ml 70% EtOH were added, and the seeds were stirred for 5 min. The EtOH was discarded and replaced by 50 ml 1.5% sodium hypochlorite. The seeds were stirred for an additional 45 - 60 min on a magnetic stirrer. The sodium hypochlorite was then discarded and the seeds rinsed 3 to 4 times with autoclaved ddH_2O. Finally 30 ml of ddH_2O were added, and seeds incubated over night at 10 - 15°C in an incubator.

Isogenic transformation of white clover

The seed coat and endosperm layer of the white clover seeds prepared as above were removed with a pair of 18 G or 21 G needles. The cotyledons were cut from the hypocotyl leaving a ca. 1.5 mm piece of the cotyledon stalk. One of the pair of cotyledons was not transformed and transferred straight to its grid position on a 20 cm petri dish containing regeneration medium. The other cotyledon was transferred to a well on the 96 well plate containing ddH_2O. After finishing the isolation of clover cotyledons, ddH_2O in the wells was replaced with *Agrobacterium* suspension (diluted to an $OD_{600} = 0.2 - 0.4$). The 96 well plate was sealed with its lid and incubated for 40 min at room temperature.

After the incubation period, each cotyledon was transferred to paper towel using the small dissection needle, dried and plated onto regeneration medium RM73. The plates were then incubated at 25°C with a 16h light/8h dark photoperiod. On day 4, the explants were transferred to fresh regeneration medium. Cotyledons transformed with *Agrobacterium* were transferred to RM73 containing cefotaxime (antibacterial agent) and gentamycin. The dishes were sealed with Parafilm and incubated at 25°C under a 16/8 h photoperiod. Explants were subcultured every three weeks for a total of nine weeks onto fresh RM 73 containing cefotaxime and gentamycin. Shoots with a green base were then transferred to root-inducing medium RIM. Roots developed after 1 – 3 weeks, and plantlets were transferred to soil when the roots were well established.

Preparation of genomic DNA

3 – 4 leaves of white clover plants regenerated on selective medium were harvested and freeze-dried. The tissue was homogenised on a Retsch MM300

mixer mill, then centrifuged for 10 min at 1700xg to collect cell debris. Genomic DNA was isolated from the supernatant using Wizard Magnetic 96 DNA Plant System kits (Promega) on a Biomek FX (Beckman Coulter). 5 µl of the sample (50 µl) were then analysed on an agarose gel to check the yield and the quality of the genomic DNA.

Analysis of DNA using real-time PCR

Genomic DNA was analysed for the presence of the transgene by real-time PCR using SYBR Green chemistry. PCR primer pairs (Table 4) were designed using MacVector (Accelrys). The forward primer was located within the $35S^2$ promoter region and the reverse primer within the transgene to amplify products of approximately 150 - 250 bp as recommended. The positioning of the forward primer within the $35S^2$ promoter region guaranteed that endogenous genes in white clover were not detected.

5 µl of each genomic DNA sample was run in a 50 µl PCR reaction including SYBR Green on an ABI (Applied Biosystems) together with samples containing DNA isolated from wild type white clover plants (negative control), samples containing buffer instead of DNA (buffer control) and samples containing the plasmid used for transformation (positive plasmid control).

Plants were obtained after transformation with all chimeric constructs and selection on medium containing gentamycin.

TABLE 4

List of primers used for Real-time PCR analysis of white clover plants transformed with chimeric white clover genes involved in condensed tannin biosynthesis

construct	primer 1 (forward), 5'->3'	primer 2 (reverse), 5'->3'
pPZP221TrCHSa3	CATTTCATTTGGAGAGGACACGC	AACACGGTTTGGTGGATTTGC
pPZP221TrCHSc	TTGGAGAGGACACGCTGAAATC	ACAAGTTGGTGAGGGAATGCC
pPZP221TrCHSf	CATTTCATTTGGAGAGGACACGC	TCGTTGCCTTTCCCTGAGTAGG
pPZP221TrCHSh	TCATTTGGAGAGGACACGCTG	CGGTCACCATTTTTTTGTTGGAGG
pPZP221TrBANa	TTGGAGAGGACACGCTGAAATC	GCAACAAAACCAGTGCCACC
pPZP221TrLARa	ATGACGCACAATCCCACTATCC	TTAGAAGAGAGAAGAGGTCCTGGC
pPZP221TrLARb	ATGACGCACAATCCCACTATCC	TTAGAAGAGAGAAGAGGTCCTGGC
pPZP221TrLARc	ATGACGCACAATCCCACTATCC	TTAGAAGAGAGAAGAGGTCCTGGC

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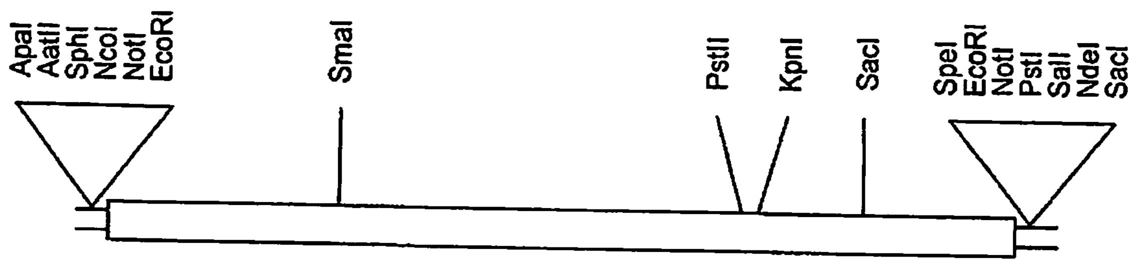
Finally, it is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

It will also be understood that the term "comprises" (or its grammatical variants) as used in this specification is equivalent to the term "includes" and should not be taken as excluding the presence of other elements or features.

Documents cited in this specification are for reference purposes only and their inclusion is not acknowledgment that they form part of the common general knowledge in the relevant art.

Agriculture Victoria Services Pty Ltd
AgResearch Limited
By their Registered Patent Attorneys
Freehills Carter Smith Beadle

14 August 2003



TrCHSa3

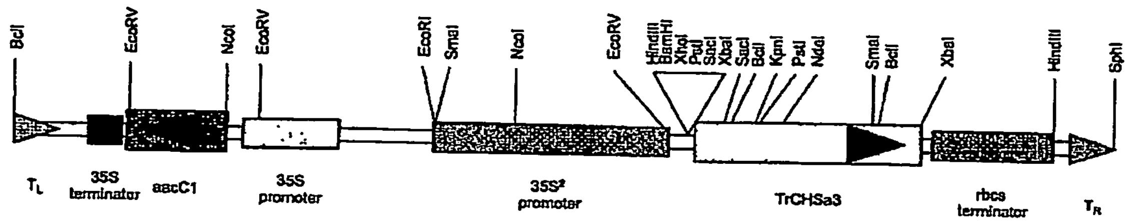
FIGURE 1

```
   1 GAATTCACTA GTGATTAAGC AGTGGTAACA ACGCAGAGTA CGCGGGGAAC
  51 AAAAACAACT ACGCATATTA TATATATATA TATATAGTCT ATAATTGAAA
 101 GAAACTGCTA AAGATATTAT TAAGATATGG TGAGTGTAGC TGAAATTCGC
 151 AAGGCTCAGA GGGCTGAAGG CCCTGCAACC ATTTTGGCCA TTGGCACTGC
 201 AAATCCACCA AACCGTGTTG AGCAGAGCAC ATATCCTGAT TTCTACTTCA
 251 AAATTACAAA CAGTGAGCAC AAGACTGAGC TCAAAGAGAA GTTCCAACGC
 301 ATGTGTGACA AATCCATGAT CAAGAGCAGA TACATGTATC TAACAGAAGA
 351 GATTTTGAAA GAAAATCCTA GTCTTTGTGA ATACATGGCA CCTTCATTGG
 401 ATGCTAGGCA AGACATGGTG GTGGTTGAGG TACCTAGACT TGGGAAGGAG
 451 GCTGCAGTCA AGGCCATTAA AGAATGGGGT CAACCAAAGT CAAAGATTAC
 501 TCACTTAATC TTTTGCACCA CAAGTGGTGT TGACATGCCT GGTGCTGATT
 551 ACCAACTCAC AAAACTCTTA GGTCTTCGCC CATATGTGAA AAGGTATATG
 601 ATGTACCAAC AAGGTTGTTT TGCAGGAGGC ACGGTGCTTC GTTTGGCAAA
 651 AGATTTGGCC GAGAACAACA AAGGTGCTCG TGTGCTAGTT GTTTGTTCTG
 701 AAGTCACCGC AGTCACATTT CGCGGCCCCA GTGATACTCA CTTGGACAGT
 751 CTTGTTGGAC AAGCATTGTT TGGAGATGGA GCCGCTGCAC TAATTGTTGG
 801 TTCTGATCCA GTGCCTGAAA TTGAGAAACC AATATTTGAG ATGGTTTGGA
 851 CTGCACAAAC AATTGCTCCA GACAGTGAAG GTGCCATTGA TGGTCATCTT
 901 CGTGAAGCTG GGCTAACATT TCATCTTCTT AAAGATGTTC CTGGGATTGT
 951 ATCAAAGAAC ATTAATAAAG CATTGGTTGA GGCTTTCCAA CCATTAGGAA
1001 TTTCTGACTA CAACTCAATC TTTTGGATTG CACACCCGGG TGGACCTGCA
1051 ATTCTTGATC AAGTAGAACA AAAGCTAGCC TTGAAGCCCG AAAAGATGAG
1101 GGCCACGAGG GAAGTTCTAA GTGAATATGG AAACATGTCA AGCGCATGTG
1151 TATTGTTCAT CTTAGATGAG ATGCGGAAGA AATCGGCTCA AAATGGACTT
1201 AAGACAACTG GAGAAGGACT TGATTGGGGT GTGTTGTTCG GCTTCGGACC
1251 AGGACTTACC ATTGAAACCG TTGTTCTTCG TAGCGTGGCT ATATAAGATG
1301 TGTGATTGTT TTTATTTTAA TGTATTACTT TTAATCTTGC TGCCTTGAAT
1351 TTCGATTTAA GAATAAATAA ATATATCTTT TGATAAAAAA AAAAAAAAAA
1401 AAAAAAAAAA AAGTACTCTG CGTTGTTACC ACTGCTTAAT CGAATTC
```

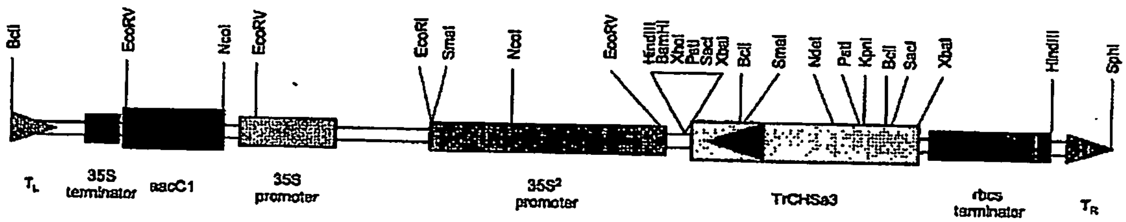
FIGURE 2

```
  1 MVSVAEIRKA QRAEGPATIL AIGTANPPNR VEQSTYPDFY FKITNSEHKT
 51 ELKEKFQRMC DKSMIKSRYM YLTEEILKEN PSLCEYMAPS LDARQDMVVV
101 EVPRLGKEAA VKAIKEWGQP KSKITHLIFC TTSGVDMPGA DYQLTKLLGL
151 RPYVKRYMMY QQGCFAGGTV LRLAKDLAEN NKGARVLVVC SEVTAVTFRG
201 PSDTHLDSLV GQALFGDGAA ALIVGSDPVP EIEKPIFEMV WTAQTIAPDS
251 EGAIDGHLRE AGLTFHLLKD VPGIVSKNIN KALVEAFQPL GISDYNSIFW
301 IAHPGGPAIL DQVEQKLALK PEKMRATREV LSEYGNMSSA CVLFILDEMR
351 KKSAQNGLKT TGEGLDWGVL FGFGPGLTIE TVVLRSVAI
```

FIGURE 3

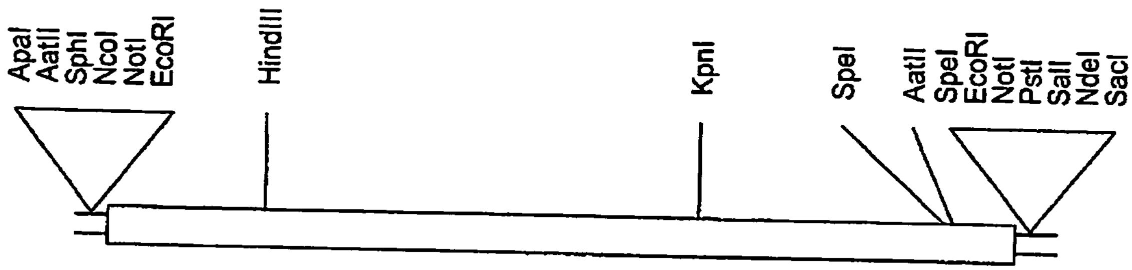


pPZP221:35S²TrCHSa3 sense



pPZP221:35S²TrCHSa3 anti

FIGURE 4



TrCHSc

FIGURE 5

1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG GATTCAATCT
51 GTTGTGCATA AAATTCACTC ATTGCATAGA AAACCATACA CATTTGATCT
101 TGCAAAGAAG AAATATGGGA GACGAAGGTA TAGTGAGAGG TGTCACAAAG
151 CAGACAACCC CTGGGAAGGC TACTATATTG GCTCTTGGCA AGGCATTCCC
201 TCACCAACTT GTGATGCAAG AGTGTTTAGT TGATGGTTAT TTTAGGGACA
251 CTAATTGTGA CAATCCTGAA CTTAAGCAGA AACTTGCTAG ACTTTGTAAG
301 ACAACCACGG TAAAAACAAG GTATGTTGTT ATGAATGAGG AGATACTAAA
351 GAAATATCCA GAACTTGTTG TCGAAGGCGC CTCAACTGTA AAACAACGTT
401 TAGAGATATG TAATGAGGCA GTAACACAAA TGGCAATTGA AGCTTCCCAA
451 GTTTGCCTAA AGAATTGGGG TAGATCCTTA TCGGACATAA CTCATGTGGT
501 TTATGTTTCA TCTAGTGAAG CTAGATTACC CGGTGGTGAC CTATACTTGT
551 CAAAAGGACT AGGACTAAAC CCTAAAATTC AAAGAACCAT GCTCTATTTC
601 TCTGGATGCT CGGGAGGCGT AGCCGGCCTT CGCGTTGCGA AAGACGTAGC
651 TGAGAACAAC CCTGGAAGTA GAGTTTTGCT TGCTACTTCG GAAACTACAA
701 TTATTGGATT CAAGCCACCA AGTGTTGATA GACCTTATGA TCTTGTTGGT
751 GTGGCACTCT TTGGAGATGG TGCTGGTGCA ATGATAATTG GCTCAGACCC
801 GGTATTTGAA ACTGAGACAC CATTGTTTGA GCTGCATACT TCAGCTCAGG
851 AGTTTATACC AGACACCGAG AAGAAAATTG ATGGGCGGCT GACGGAGGAG
901 GGCATAAGTT TCACACTAGC AAGGGAACTT CCGCAGATAA TCGAAGACAA
951 TGTTGAGGGA TTCTGTAATA AACTAATTGA TGTTGTTGGG TTGGAGAATA
1001 AGGAGTACAA TAAGTTGTTT TGGGCTGTGC ATCCAGGTGG GCCTGCGATA
1051 TTGAATCGCG TGGAGAAGCG GCTTGAGTTG TCGCCGCAGA AGCTGAATGC
1101 TAGTAGAAAA GCTCTAATGG ATTATGGAAA TGCTAGCAGC AATACTATTG
1151 TTTATGTGCT GGAATATATG CTAGAAGAGG AAAAGAAGAT TAAAAAGGCG
1201 GGTGGAGGAG ATTCTGAATG GGGATTGATA CTTGCTTTTG GACCTGGAAT
1251 TACTTTTGAG GGGATTCTAG CAAGGAACTT GTGTGCATGA AGTCTTATAC
1301 AATTGTGATG CATGACTTAT ACTCTTATTT CTACTAATTA TTATATTAAG
1351 CAAATTCAGA ACTTTTAAGT AATGATTTAA TGAAGAATAC TTATAGTATA
1401 TTGACTTTAT TCACTTTCAA AGCAAGTTTA TGATCCTAAG ACATGGTAGA
1451 ACTTGAGCAT GTGGAATAGT TGTAACAAAA ACTCTAAGCA AATAGAGACT
1501 TTATGTAGTA TAAAGCATTT CCAGACATGA TAAATAATGG TACCTCAGAA
1551 CATAAAATAT ATTTAGCTAT CTTTCATCCC CAACTTTACA CATCCACCAA
1601 GGTACAGAAT AAGCATATGT CAACACAAAA TGTACTCTAA GTCTAACATG
1651 AGTAACCAAA CATGATGCCT GATTAAGTTA AAAGAAAAGA AAATCTGAGG
1701 GCATAGATCT TCAATCACAC CACTCCAGAG GGAAGGCGTA GAACAAGCTG

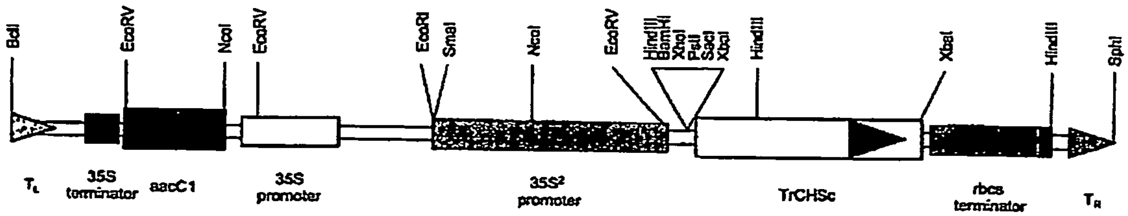
FIGURE 6

1751 TCCGCCGAAA ACACTGCAAT TCAATAAATA TCATTAGGAC AACAGTGCAG
1801 AGTCATGCGG GAAATGTCTT AAGTCACTGT ACTAAAAATA TAGGATTATA
1851 TTATGAACTA TACTAACCTT TTCACATAAT AGTAACAGAA ATCAGCTAAG
1901 ATGAATGTCT GGACAATTTC TGAGATAAGA ACCATGACGG CCATAAGCCA
1951 TACCCCAAGG CAACCAATAA ATGTCCACGG GTATCTAACA CCTGTTGCAA
2001 GAAATAGTAA GTTATTAGGA GATGTGCGGT TACGAAATTC AAGCTACACA
2051 ACAAAAGGAG GCCAGAACAA CAGCAATCTT GTAACCAGAT GACAACAATA
2101 AAATGTAAAC TTAAAGAGAC CGAACACACA AACATTGCAA CTCAGATGGA
2151 ATTGCTGCCA TGTAACTAGT AGGAGATTTG GGACGTCAAA TCAGTATATT
2201 ATGCAAATAC AAGGTATGAC CGCCTTGTCT ATTGTAGCAT ACAACAAACG
2251 TACAGTGGGT TTGTCCCTCT CAAAATGGCA GGATCTTTAC AGCACAATAT
2301 TTGGTTTTGT CATACTTATA CCATAAAAAA AAAAAAAAAA AAAAAAAAAA
2351 AAAGTACTCT GCGTTGTTAC CACTGCTTAA TCACTAGTGA ATTC

FIGURE 6 (cont.)

1	MGDEGIVRGV	TKQTTPGKAT	ILALGKAFPH	QLVMQECLVD	GYFRDTNCDN
51	PELKQKLARL	CKTTTVKTRY	VVMNEEILKK	YPELVVEGAS	TVKQRLEICN
101	EAVTQMAIEA	SQVCLKNWGR	SLSDITHVVY	VSSSEARLPG	GDLYLSKGLG
151	LNPKIQRTML	YFSGCSGGVA	GLRVAKDVAE	NNPGSRVLLA	TSETTIIGFK
201	PPSVDRPYDL	VGVALFGDGA	GAMIIGSDPV	FETETPLFEL	HTSAQEFIPD
251	TEKKIDGRLT	EEGISFTLAR	ELPQIIEDNV	EGFCNKLIDV	VGLENKEYNK
301	LFWAVHPGGP	AILNRVEKRL	ELSPQKLNAS	RKALMDYGNA	SSNTIVYVLE
351	YMLEEEKKIK	KAGGGDSEWG	LILAFGPGIT	FEGILARNLC	A

FIGURE 7



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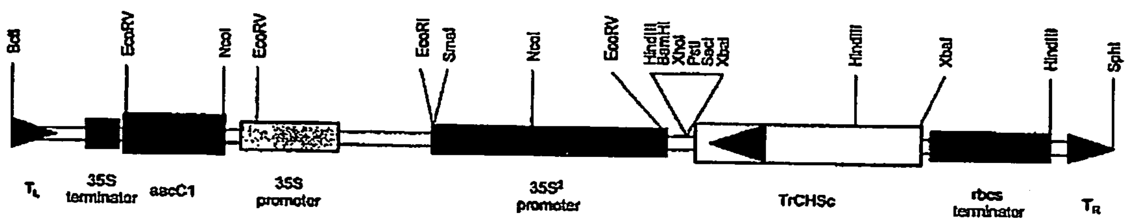
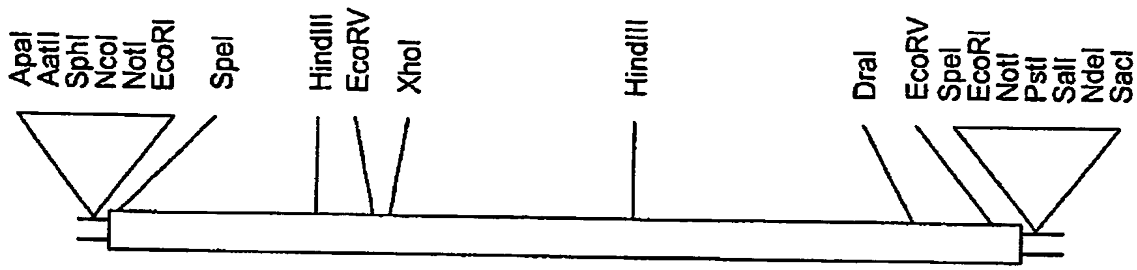


pPZP221:35S²TrCHSc anti

FIGURE 8



TrCHSf

FIGURE 9

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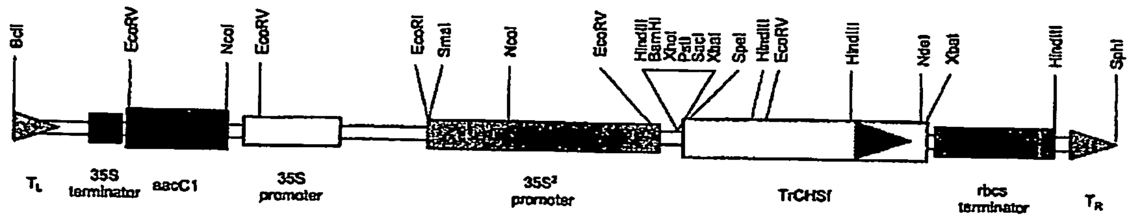
   1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG ACTAAGCCTT
  51 GATTCATTGT TTGTTTCCAT AACACAAGAA CTAGTGTTTG CTTGAATCTT
 101 AAGAAAAAAT GCCTCAAGGT GATTTGAATG GAAGTTCCTC GGTGAATGGA
 151 GCACGTGCTA GACGTGCTCC TACTCAGGGA AAGGCAACGA TACTTGCATT
 201 AGGAAAGGCT TTCCCCGCCC AGGTCCTCCC TCAAGAGTGC TTGGTGGAAG
 251 GATTCATTCG CGACACTAAG TGTGACGATA CTTATATTAA GGAGAAATTG
 301 GAGCGTCTTT GCAAAAACAC AACTGTGAAA ACAAGATACA CAGTAATGTC
 351 AAAGGAGATC TTAGACAACT ATCCAGAGCT AGCCATAGAT GGAACACCAA
 401 CAATAAGGCA AAAGCTTGAA ATAGCAAATC CAGCAGTAGT TGAAATGGCA
 451 ACAAGAGCAA GCAAAGATTG CATCAAAGAA TGGGGAAGGT CACCTCAAGA
 501 TATCACACAC ATAGTCTATG TTTCCTCGAG CGAAATTCGT CTACCCGGTG
 551 GTGACCTTTA TCTTGCAAAT GAACTCGGCT TAAACAGCGA TGTTAATCGC
 601 GTAATGCTCT ATTTCCTCGG TTGCTACGGC GGTGTCACTG GCTTACGTGT
 651 CGCCAAAGAC ATCGCCGAAA ATAACCCTGG TAGTAGGGTG TTACTCACAA
 701 CATCCGAGAC CACTATTCTC GGTTTTCGAC CACCGAGTAA AGCTAGACCT
 751 TATGACCTCG TTGGCGCTGC ACTTTTCGGT GATGGCGCCG CTGCTGCAAT
 801 AATTGGAACA GACCCTATAT TGAATCAAGA ATCACCTTTC ATGGAATTGA
 851 ACCATGCAGT CCAAAAATTC TTGCCTGATA CACAAAATGT GATTGATGGT
 901 AGAATCACTG AAGAGGGTAT TAATTTTAAG CTTGGAAGAG ACCTTCCTCA
 951 AAAAATTGAA GACAATATTG AAGAATTTTG CAAGAAAATT ATGGCTAAAA
1001 GTGATGTTAA GGAATTTAAT GACTTATTTT GGGCTGTTCA TCCTGGTGGG
1051 CCAGCTATAC TCAATAAGCT AGAAAATATA CTCAAATTGA AAAGTGATAA
1101 ATTGGATTGT AGTAGGAAGG CATTAATGGA TTATGGAAAT GTTAGTAGCA
1151 ATACTATATT CTATGTGATG GAGTATATGA GAGATTATTT GAAGGAAGAT
1201 GGAAGTGAAG AATGGGGATT AGGATTGGCT TTTGGACCAG GGATTACTTT
1251 TGAAGGGGTT CTCCTCCGTA GCCTTTAATC TTGAAATAAT AATTCATATG
1301 AAATTACTTG TCTTAAGATT GTGATAGGAA GATGAATATG TATTGGATTA
1351 ATATTGATAT GGTGTTATTT TAAGTTGATT TTAAAAAAAG TTTATTAATA
1401 AAGTATGATG TAACAATTGT TGTTTGAATG TTAAAAGGGA AGTATACTAT
1451 TTTAAGTTCT TGACCATACT GATTTTTTCT TTACACATTT TCATATCTAA
1501 AATTGTTCTA TGATATCTTC ATTGTTGATA CTGTAATAAT ATAATATCTA
1551 ATTTGGCTGG CAAAATGAAA GATTTTTCAC CGAAAAAAAA AAAAAAAAAA
1601 AAAAAAAAAA AAGTACTCTG CGTTGTTACC ACTGCTTAAT CACTAGTGAA
1651 TTC
```

FIGURE 10

1 MPQGDLNGSS SVNGARARRA PTQGKATILA LGKAFPAQVL PQECLVEGFI
51 RDTKCDDTYI KEKLERLCKN TTVKTRYTVM SKEILDNYPE LAIDGTPTIR
101 QKLEIANPAV VEMATRASKD CIKEWGRSPQ DITHIVYVSS SEIRLPGGDL
151 YLANELGLNS DVNRVMLYFL GCYGGVTGLR VAKDIAENNP GSRVLLTTSE
201 TTILGFRPPS KARPYDLVGA ALFGDGAAAA IIGTDPILNQ ESPFMELNHA
251 VQKFLPDTQN VIDGRITEEG INFKLGRDLP QKIEDNIEEF CKKIMAKSDV
301 KEFNDLFWAV HPGGPAILNK LENILKLKSD KLDCSRKALM DYGNVSSNTI
351 FYVMEYMRDY LKEDGSEEWG LGLAFGPGIT FEGVLLRSL

FIGURE 11



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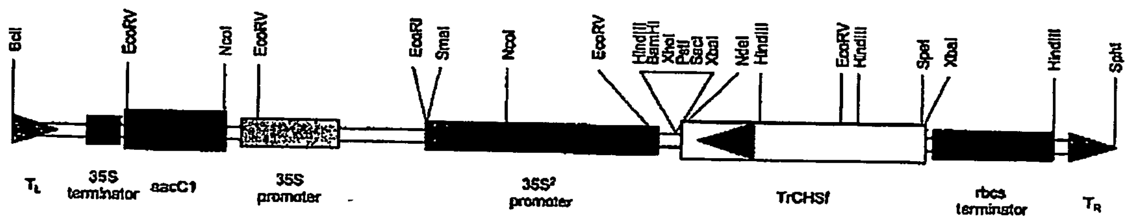
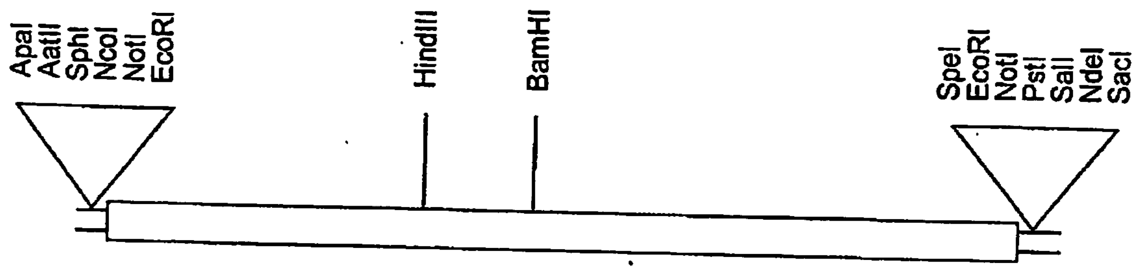


pPZP221:35S²TrCHSf anti

FIGURE 12



TrCHSh

FIGURE 13

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   1 GAATTCACTA GTGATTAAGC AGTGGTAACA ACGCAGAGTA CGCGGGGGAA
  51 TCCACCAAAT CAACACCATT AATAACCTTC CAAATTCTCG TTACCTCACC
 101 AAATCTCATT TTTCATTATA TATCTTGGGT ACATCTTTTG TTACCTCCAA
 151 CAAAAAAATG GTGACCGTAG AAGAGATTCG TAACGCCCAA CGTTCAAATG
 201 GCCCTGCCAC TATCTTAGCT TTTGGCACAG CCACTCCTTC TAACTGTGTC
 251 ACTCAAGCTG ATTATCCTGA TTACTACTTT CGTATCACCA ACAGCGAACA
 301 TATGACTGAT CTTAAGGAAA AATTCAAGCG GATGTGTGAT AGATCAATGA
 351 TAAAGAAACG TTACATGCAC CTAACAGAAG ACTTTCTGAA GGAGAATCCA
 401 AATATGTGTG AATACATGGC ACCATCACTA GATGTAAGAC GAGACATAGT
 451 GGTTGTTGAA GTACCAAAGC TAGGTAAAGA AGCAGCAAAA AAAGCCATAT
 501 GTGAATGGGG ACAACCAAAA TCCAAAATCA CACATCTTGT TTTCTGCACC
 551 ACTTCCGGTG TTGACATGCC GGGAGCCGAT TACCAACTCA CCAAACTTTT
 601 AGGCTTAAAA CCTTCTGTCA AGCGTCTCAT GATGTATCAA CAAGGTTGTT
 651 TCGCTGGCGG CACAGTTCTC CGCTTAGCAA AAGACCTTGT TGAGAATAAC
 701 AAAAATGCAA GAGTTCTTGT TGTTTGTTCT GAAATTACTG CGGTTACTTT
 751 TCGTGGACCA TCGGATACTC ATCTTGATTC GCTCGTGGGA CAGGCGCTTT
 801 TTGGTGATGG AGCCGCAGCA ATGATTATTG GTGCGGATCC TGATTTAACC
 851 GTGGAGCGTC CGATTTTCGA GATTGTTTCG GCTGCTCAGA CTATTCTTCC
 901 TGATTCTGAT GGCGCAATTG ATGGACATCT TCGTGAAGTG GGCTCACTT
 951 TTCATTTATT GAAAGATGTT CCGGGGATTA TTTCAAAGAA CATTGAAAAA
1001 AGTTTAGTTG AAGCTTTTGC GCCTATTGGG ATTAATGATT GGAACTCAAT
1051 ATTTTGGGTT GCACATCCAG GTGGACCGGC TATTTTAGAC CAGGTTGAAG
1101 AGAAACTCCA TCTTAAAGAG GAGAAACTCC GGTCCACCCG GCATGTGCTT
1151 AGTGAATATG GAAATATGTC AAGTGCATGT GTTTTATTTA TTTTGGATGA
1201 AATGAGAAAG AGGTCTAAAG AGGAAGGGAT GATTACAACT GGTGAAGGGT
1251 TGGAATGGGG TGTGTTGTTT GGGTTTGGAC CGGGTTTAAC TGTTGAAACC
1301 GTTGTGCTTC ATAGTGTTCC GGTTCAGGGT TGAATTTATT ATACATAGAT
1351 TGGAAAATAA AATTTGCCTG CCGAGAGATG TGAACTAACT TTGTAGGCAA
1401 GCTCAAATTA AAGTTTGAGA TAATATTGTG CTTTAGTTAT TATGGTATGT
1451 AATGTAATGT TTTTACTTTT TTCGAAATTC ATGTAATTTG ATATGTAAAG
1501 TAATATGTTT GGGTTGGAAT ATAATTATTT GTTAACTAAA AAAAAAAAAA
1551 AAAAAAAAAA AAAAAGTACT CTGCGTTGTT ACCACTGCTT AATCGAATTC
```
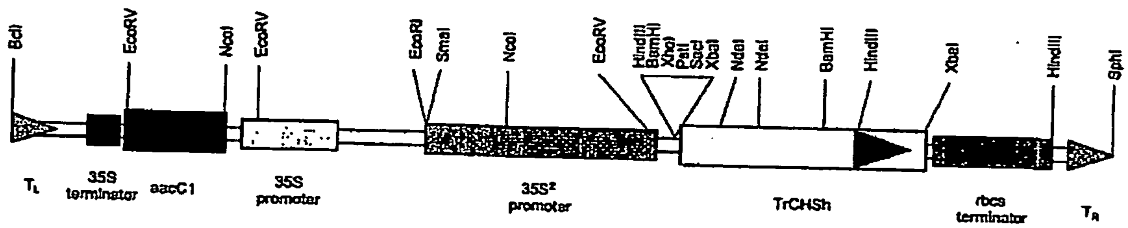

FIGURE 14

1 MVTVEEIRNA QRSNGPATIL AFGTATPSNC VTQADYPDYY FRITNSEHMT
51 DLKEKFKRMC DRSMIKKRYM HLTEDFLKEN PNMCEYMAPS LDVRRDIVVV
101 EVPKLGKEAA KKAICEWGQP KSKITHLVFC TTSGVDMPGA DYQLTKLLGL
151 KPSVKRLMMY QQGCFAGGTV LRLAKDLVEN NKNARVLVVC SEITAVTFRG
201 PSDTHLDSLV GQALFGDGAA AMIIGADPDL TVERPIFEIV SAAQTILPDS
251 DGAIDGHLRE VGLTFHLLKD VPGIISKNIE KSLVEAFAPI GINDWNSIFW
301 VAHPGGPAIL DQVEEKLHLK EEKLRSTRHV LSEYGNMSSA CVLFILDEMR
351 KRSKEEGMIT TGEGLEWGVL FGFGPGLTVE TVVLHSVPVQ G

FIGURE 15



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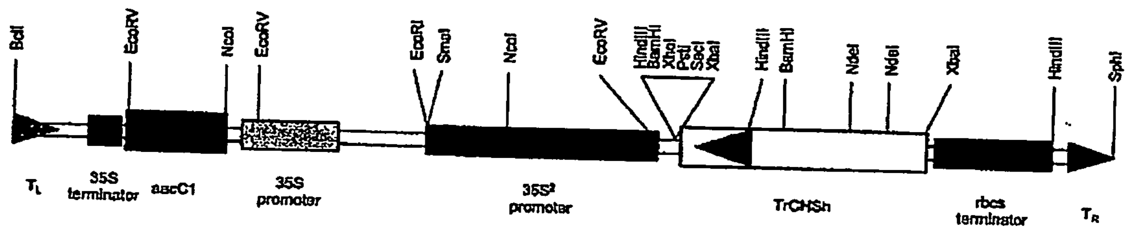
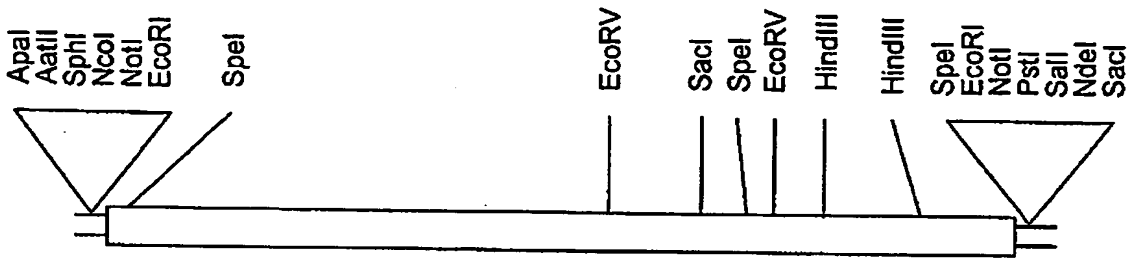


pPZP221:35S²TrCHSh anti

FIGURE 16



TrBANa

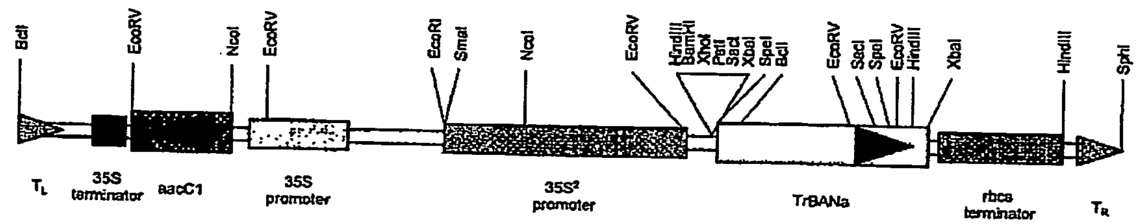
FIGURE 17

1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG ATAAAAACTG
51 CACTAGTGTG TATAAGTTTC TTGGTGAAAA AAGAGTTTGT AAATTAACAT
101 CATGGCTAGT ATCAAACAAA TTGGAAACAA GAAAGCATGT GTGATTGGTG
151 GCACTGGTTT TGTTGCATCT ATGTTGATCA AGCAGTTACT TGAAAAGGGT
201 TATGCTGTTA ATACTACCGT TAGAGACCCA GATAGCCCTA AGAAAATATC
251 TCACCTAGTG GCACTGCAAA GTTTGGGGGA ACTGAATCTA TTTAGAGCAG
301 ACTTAACAGT TGAAGAAGAT TTTGATGCTC CTATAGCAGG ATGTGAACTT
351 GTTTTTCAAC TTGCTACACC TGTGAACTTT GCTTCTCAAG ATCCTGAGAA
401 TGACATGATA AAGCCAGCAA TCAAAGGTGT GTTGAATGTG TTGAAAGCAA
451 TTGCAAGAGC AAAAGAAGTT AAAAGAGTTA TCTTAACATC TTCGGCAGCC
501 GCGGTGACTA TAAATGAACT CAAAGGGACA GGTCATGTTA TGGATGAAAC
551 CAACTGGTCT GATGTTGAAT TTCTCAACAC TGCAAAACCA CCCACTTGGG
601 GTTATCCTGC CTCAAAAATG CTAGCTGAAA AGGCTGCATG GAAATTTGCT
651 GAAGAAAATG ACATTGATCT AATCACTGTG ATACCTAGTT TAACAACTGG
701 TCCTTCTCTC ACACCAGATA TCCCATCTAG TGTTGGCTTG GCAATGTCTC
751 TAATAACAGG CAATGATTTT CTCATAAATG CTTTGAAAGG AATGCAGTTT
801 CTGTCGGGTT CGTTATCCAT CACTCATGTT GAGGATATTT GCCGAGCTCA
851 TATATTTCTT GCAGAGAAAG AATCAGCTTC TGGTAGATAC ATTTGCTGTG
901 CTCACAATAC TAGTGTTCCC GAGCTTGCAA AGTTTCTCAA CAAACGATAT
951 CCTCAGTATA AAGTTCCAAC TGAATTTGAT GATTGCCCCA GCAAGGCAAA
1001 GTTGATAATC TCTTCTGAAA AGCTTATCAA AGAAGGGTTC AGTTTCAAGC
1051 ATGGTATTGC CGAAACTTTC GACCAGACTG TCGAGTATTT TAAGACTAAG
1101 GGGGCACTGA AGAATTAGAT TTTGATATTT CTAATTCAAT AGCAAACTCT
1151 AAGCTTGTTA TGTGTTTGTG AAGTTCAGAG TGAAATATCA AATGAATAAG
1201 TGGAGAGAGC ACAATAAGAG GAGAGCACAA TAATTTTGGA AAAAAAAAAA
1251 AAAAAAAAAA AAAAAAAAGT ACTCTGCGTT GTTACCACTG CTTAATCACT
1301 AGTGAATTC

FIGURE 18

1 MASIKQIGNK KACVIGGTGF VASMLIKQLL EKGYAVNTTV RDPDSPKKIS
51 HLVALQSLGE LNLFRADLTV EEDFDAPIAG CELVFQLATP VNFASQDPEN
101 DMIKPAIKGV LNVLKAIARA KEVKRVILTS SAAAVTINEL KGTGHVMDET
151 NWSDVEFLNT AKPPTWGYPA SKMLAEKAAW KFAEENDIDL ITVIPSLTTG
201 PSLTPDIPSS VGLAMSLITG NDFLINALKG MQFLSGSLSI THVEDICRAH
251 IFLAEKESAS GRYICCAHNT SVPELAKFLN KRYPQYKVPT EFDDCPSKAK
301 LIISSEKLIK EGFSFKHGIA ETFDQTVEYF KTKGALKN

FIGURE 19



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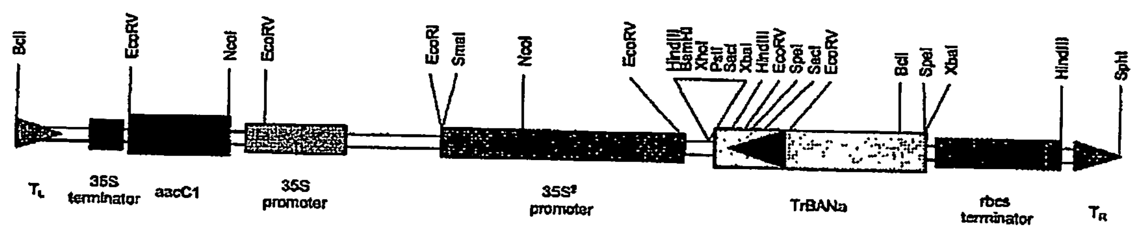

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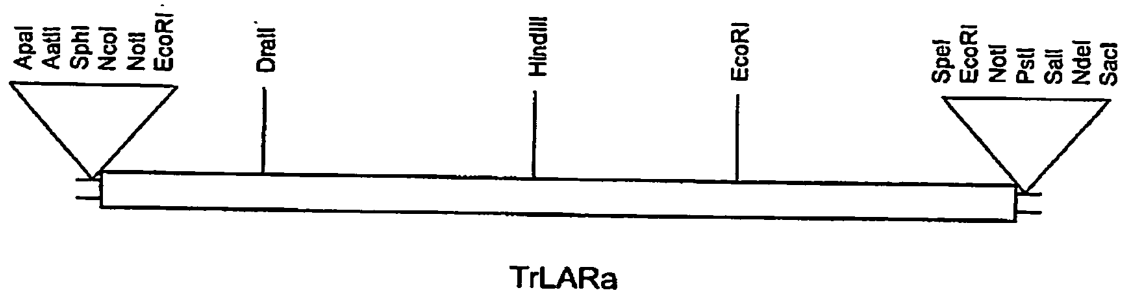
FIGURE 20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NcoI
NotI
EcoRI
DraII
HindIII
EcoRI
SpeI
EcoRI
NotI
PstI
SalI
NdeI
SacI
TrLARa

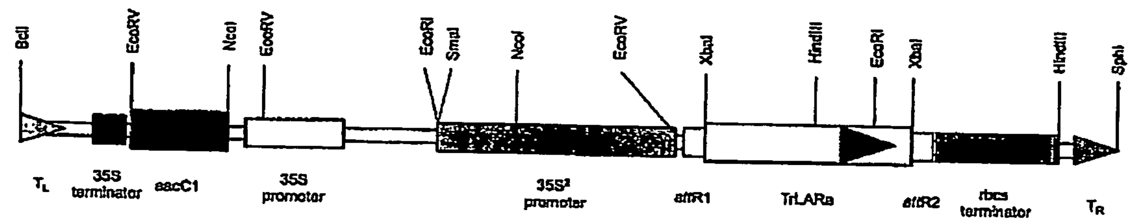
FIGURE 21

1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG GATACCAACA
51 TTGTCACAAT TAACTCTAAA AGCAAAGCAA TGGCACCAGC AGCAACATCA
101 TCACCAACCA CTCCTACTAC TACCAAGGGT CGTGTCCTAA TTGTTGGAGG
151 AACAGGTTTC ATTGGAAAAT TTGTAACTGA GGCAAGTCTT TCCACAACAC
201 ACCCAACCTA CTTGTTGGTT CGGCCAGGAC CTCTTCTCTC TTCTAAGGCT
251 GCCACTATTA AGGCATTCCA AGAGAAAGGT GCCATTGTCA TTTATGGTCG
301 GGTAAATAAT AAGGAGTTCA TGGAGATGAT TTTGAAAAAG TATGAGATAA
351 ATGTAGTCAT TTCTGCAATA GGAGGCTCTG ATGGCTTGCT GGAACAGCTT
401 ACTTTGGTGG AGGCCATGAA ATCTATTAAC ACCATTAAGA GGTTTTTGCC
451 TTCGGAATTT GGTCACGATG TGGACAGAGC AAATCCTGTG GAACCTGGCC
501 TAACAATGTA CAAACAGAAA CGTTTGGTTA GACGTGTGAT CGAAGAATCT
551 GGTATACCAT ACACCTACAT CTGTTGCAAT TCGATCGCAT CTTGGCCGTA
601 CTATGACAAT TGTCATCCAT CACAGCTTCC TCCACCGTTG GATCAATTAC
651 ATATTTATGG TCATGGCGAT GTCAAAGCTT ACTTTGTTGA TGGCTATGAT
701 ATTGGGAAAT TCACAATGAA GGTCATTGAT GATGAAAGAA CAATCAACAA
751 AAATGTTCAT TTTCGACCTT CTAACAATTG TTATAGCATG AATGAGCTTG
801 CTTCTTTGTG GGAAAACAAA ATTGCACGAA AAATTCCTAG AGTGATCGTC
851 TCTGAAGACG ATCTTCTAGC AATAGCCGCA GAAAATTGCA TACCGGAAAG
901 TGTCGTGGCA CCAATCACTC ATGATATATT CATCAATGGA TGTCAAGTTA
951 ACTTCAAGAT AGATGGAATT CATGATGTTG AAATTGGCAC TCTATATCCT
1001 GGTGAATCGG TAAGAAGTTT GGAGGAATGC TATGAGAAAT TTGTTGTCAT
1051 GGCGGCTGAC AAGATTCATA AAGAAGAAAC TGGAGTTACC GCAGGTGGGG
1101 GCGGCACAAC GGCTATGGTA GAGCCGGTGC CAATCACAGC TTCCTGTTGA
1151 AAAGGTTCAC CTGAGGTGGA TATTCTTTTG AGTCATAAGA CATGTTGATT
1201 GTTGATGTTG TTTTCAAGAA TGTTTCATCA TTTCATGTGT TTTATTAATC
1251 CTAAGTACAA ATAATTGCTG TCTACGTACG TTCTTAGTTG CAAAAATTCT
1301 TGTTATTCTC TATTGAGGTA AAAGTCTTCA TGTTTACAAA AAAAAAAAAA
1351 AAAAAAAAAA AAAAAAAAGT ACTCTGCGTT GTTACCACTG CTTAATCACT
1401 AGTGAATTC

FIGURE 22

1 MAPAATSSPT TPTTTKGRVL IVGGTGFIGK FVTEASLSTT HPTYLLVRPG
51 PLLSSKAATI KAFQEKGAIV IYGRVNNKEF MEMILKKYEI NVVISAIGGS
101 DGLLEQLTLV EAMKSINTIK RFLPSEFGHD VDRANPVEPG LTMYKQKRLV
151 RRVIEESGIP YTYICCNSIA SWPYYDNCHP SQLPPPLDQL HIYGHGDVKA
201 YFVDGYDIGK FTMKVIDDER TINKNVHFRP SNNCYSMNEL ASLWENKIAR
251 KIPRVIVSED DLLAIAAENC IPESVVAPIT HDIFINGCQV NFKIDGIHDV
301 EIGTLYPGES VRSLEECYEK FVVMAADKIH KEETGVTAGG GGTTAMVEPV
351 PITASC

FIGURE 23



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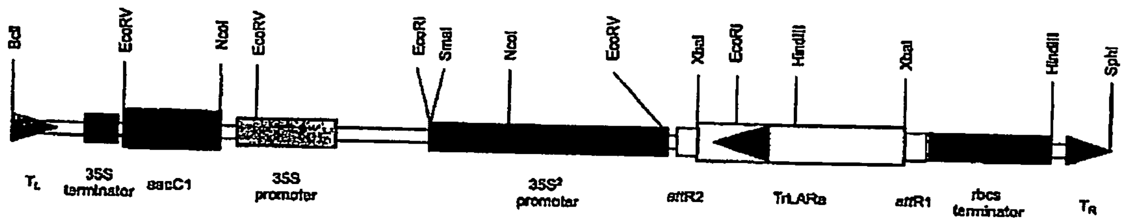

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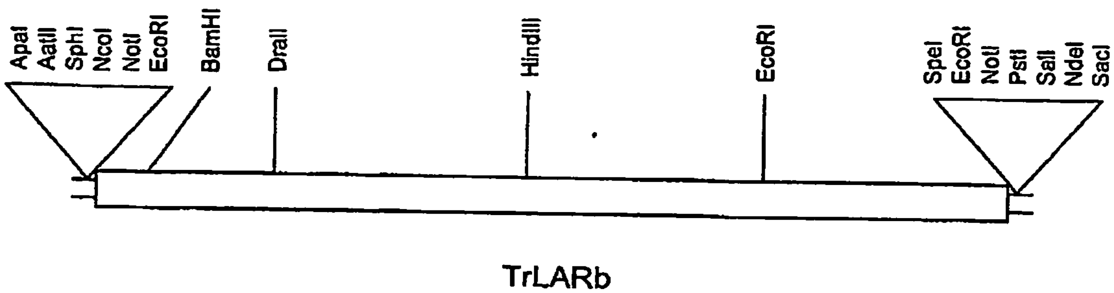
FIGURE 24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NotI
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DraII
HindIII
EcoRI
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NotI
PstI
SalI
NdeI
SacI
TrLARb

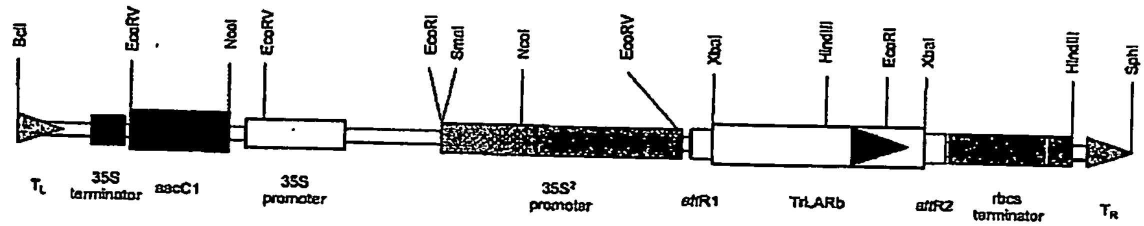
FIGURE 25

```
   1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG AGGATCCTTC
  51 CATTTTGCAT ACCAACATTG TCACAATTAA CTCTAAAAGC AAAGCAATGG
 101 CACCAGCAGC AACATCATCA CCAACCACTC CTACTACTAC CAAGGGTCGT
 151 GTCCTAATTG TTGGAGGAAC AGGTTTCATT GGAAAATTTG TAACTGAGGC
 201 AAGTCTTTCC ACAACACACC CAACCTACTT GTTGGTTCGG CCAGGACCTC
 251 TTCTCTCTTC TAAGGCTGCC ACTATTAAGG CATTCCAAGA GAAAGGTGCC
 301 ATTGTCATTT ATGGTCGGGT AAATAATAAG GAGTTCATGG AGATGATTTT
 351 GAAAAAGTAT GAGATAAATG TAGTCATTTC TGCAATAGGA GGCTCTGATG
 401 GCTTGCTGGA ACAGCTTACT TTGGTGGAGG CCATGAAATC TATTAACACC
 451 ATTAAGAGGT TTTTGCCTTC AGAATTTGGT CACGATGTGG ACAGAGCAAA
 501 TCCTGTGGAA CCTGGCCTAA CAATGTACAA ACAGAAACGT TTGGTTAGAC
 551 GTGTGATCGA AGAATCTGGT GTACCATACA CCTACATCTG TTGCAATTCG
 601 ATCGCATCCT GGCCGTACTA TGACAATTGT CATCCATCAC AGCTTCCTCC
 651 ACCGTTGGAT CAATTACATA TTTATGGTCA TGGCGATGTC AAAGCTTACT
 701 TTGTTGATGG CTATGATATT GGGAAATTCA CAATGAAGGT CATTGATGAT
 751 GAAAGAACAA TCAACAAAAA TGTTCATTTT CGACCTTCTA ACAATTGTTA
 801 TAGCATGAAT GAGCTTGCTT CTTTGTGGGA AAACAAAATT GCACGAAAAA
 851 TTCCTAGAGT GATCGTCTCT GAAGACGATC TTCTAGCAAT AGCCGCAGAA
 901 AACTGCATAC CGGAAAGTGT TGTGGCATCA ATCACTCATG ATATATTCAT
 951 CAATGGATGT CAAGTTAACT TCAAGGTAGA TGGAATTCAT GATGTTGAAA
1001 TTGGCACTCT ATATCCTGGT GAATCGGTAA GAAGTTTGGA GGAATGCTAT
1051 GAGAAATTTG TTGTCATGGC GGCTGACAAG ATTCATAAAG AAGAAACTGG
1101 AGTTACCGCA GGTGGGGGCG GCACAACGGC TATGGTAGAG CCGGTGCCAA
1151 TCACAGCTTC CTGTTGAAAA GGTTCACCTG AGGTGGATAT TCTTTTGAGT
1201 CATAAGACAT GTTGATTGTT GATGTTGTTT TCAAGAATGT TTCATCATTT
1251 CATGTGTTTT ATTAATCCTA AGTACAAATA ATTGCTGTCT ACGTACGTTC
1301 TTAGTTGCGA AAATTCTTGT TATTCTCTAT TGGGGTAAAA GTCTTCATGT
1351 TTATTGTAGT TGTGTTGGTT TTTCATATAT GCTATTTGCA ATAATGATTT
1401 TTGTGAAGCA CTTGTGGTGT ATTTACTTAC TACTGAAAAT AATGGTTACA
1451 CAAAATATAT AAAAAAATAA AAATAAGCAA AAAAAAAAAA AAAAAAAAAA
1501 AAAAAAAAAA GTACTCTGCG TTGTTACCAC TGCTTAATCA CTAGTGAATT
1551 C
```

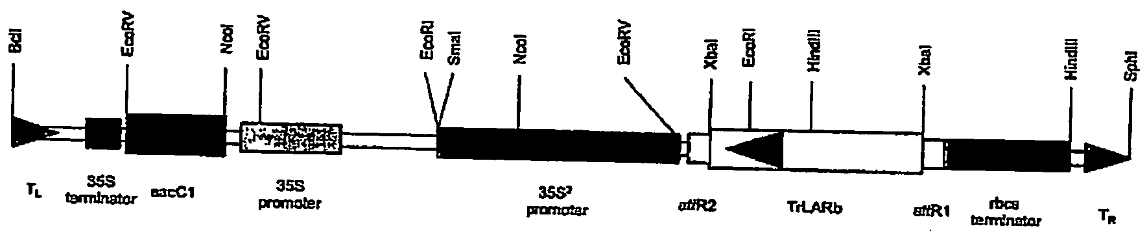
FIGURE 26

1 MAPAATSSPT TPTTTKGRVL IVGGTGFIGK FVTEASLSTT HPTYLLVRPG
51 PLLSSKAATI KAFQEKGAIV IYGRVNNKEF MEMILKKYEI NVVISAIGGS
101 DGLLEQLTLV EAMKSINTIK RFLPSEFGHD VDRANPVEPG LTMYKQKRLV
151 RRVIEESGVP YTYICCNSIA SWPYYDNCHP SQLPPPLDQL HIYGHGDVKA
201 YFVDGYDIGK FTMKVIDDER TINKNVHFRP SNNCYSMNEL ASLWENKIAR
251 KIPRVIVSED DLLAIAAENC IPESVVASIT HDIFINGCQV NFKVDGIHDV
301 EIGTLYPGES VRSLEECYEK FVVMAADKIH KEETGVTAGG GGTTAMVEPV
351 PITASC

FIGURE 27



pPZP221:35S²TrLARb sense



pPZP221:35S²TrLARb anti

FIGURE 28

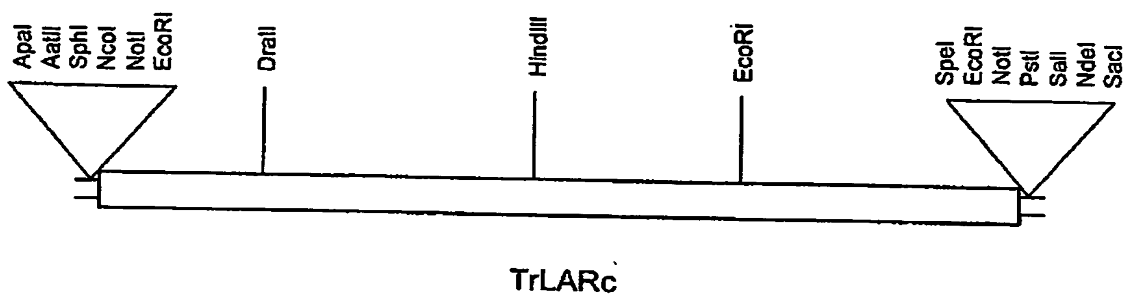

ApaI
AatII
SphI
NcoI
NotI
EcoRI
DraII
HindIII
EcoRI
SpeI
EcoRI
NotI
PstI
SalI
NdeI
SacI
TrLARc

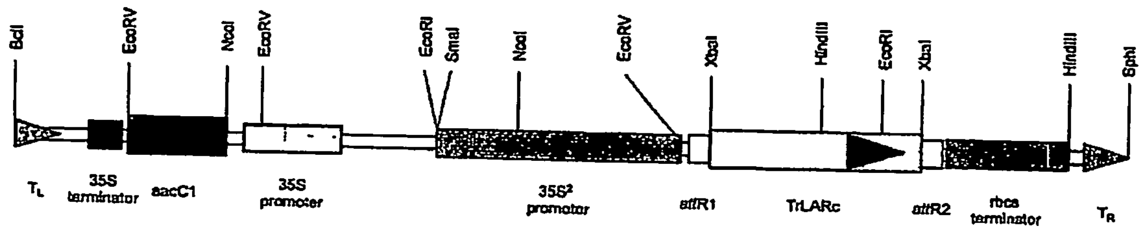
FIGURE 29

1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG GATACCAACA
51 TTGTCACAAT TAACTCTAAA AGTAAAGCAA TGGCACCAGC AGCAACATCA
101 TCACCAACCA CTCCCACTAC TACCAAGGGT CGTGTCCTAA TTGTTGGAGG
151 AACAGGTTTC ATTGGAAAAT TTGTAACTGA GGCAAGTCTT TCCACAACAC
201 ACCCAACCTA CTTGTTGGTT CGGCCAGGAC CTCTTCTCTC TTCTAAGGCT
251 GCCACTATTA AGGCATTCCA AGAGAAAGGT GCCATTGTCA TTTATGGTCG
301 GGTAAATAAT AAGGAGTTCA TGGAGATGAT TTTGAAAAAG TATGAGATAA
351 ATGTAGTCAT TTCTGCAATA GGAGGCTCTG ATGGCTTGCT GGAACAGCTT
401 ACTTTGGTGG AGGCCATGAA ATCTATTAAC ACCATTAAGA GGTTTTTGCC
451 TTCGGAATTT GGTCACGATG TGGACAGAGC AGATCCTGTG GAACCTGGCC
501 TAACAATGTA CAAACAGAAA CGTTTGGTTA GACGTGTGAT CGAAGAATCT
551 GGTATACCAT ACACCTACAT CTGTTGCAAT TCGATCGCAT CTTGGCCGTA
601 CTATGACAAT TGTCATCCAT CACAGCTTCC TCCACCGTTG GATCAATTAC
651 ATATTTATGG TCATGGCGAT GTCAAAGCTT ACTTTGTTGA TGGCTATGAT
701 ATTGGGAAAT TCACAATGAA GGTCATTGAT GATGAAAGAA CAATCAACAA
751 AAATGTTCAT TTTCGACCTT CTAACAATTG TTATAGCATG AATGAGCTTG
801 CTTCTTTGTG GGAAAACAAA ATTGCACGAA AAATTCCTAG AGTGATCGTC
851 TCTGAAGACG ATCTTCTAGC AATAGCCGCA GAAAATTGCA TACCGGAAAG
901 TGTCGTGGCA CCAATCACTC ATGATATATT CATCAATGGA TGTCAAGTTA
951 ACTTCAAGAT AGATGGAATT CATGATGTTG AAATTGGCAC TCTATATCCT
1001 GGTGAATCGG TAAGAAGTTT GGAGGAATGC TATGAGAAAT TTGTTGTCAT
1051 GGCGGCTGAC AAGATTCATA AAGAAGAAAC TGGAGTTACC GCAGGTGGGG
1101 GCGGCACAAC GGCTATGGTA GAGCCGGTGC CAATCACAGC TTCCTGTTGA
1151 AAAGGTTCAC CTGAGGTGGA TATTCTTTTG AGTCATAAGA CATGTTGATT
1201 GTTGATGTTG TTTTCAAGAA TGTTTCATCA TTTCATGTGT TTTATTAATC
1251 CTAAGTACAA ATAATTGCTG TCTACGTACG TTCTTAGTTG CAAAAATTCT
1301 TGTTATTCTC TATCAAAAAA AAAAAAAAAA AAAAAAAAAA AAAGTACTCT
1351 GCGTTGTTAC CACTGCTTAA TCACTAGTGA ATTC

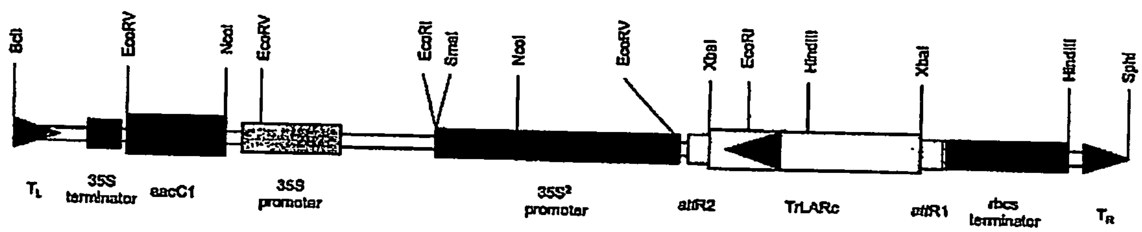
FIGURE 30

```
  1 MAPAATSSPT TPTTTKGRVL IVGGTGFIGK FVTEASLSTT HPTYLLVRPG
 51 PLLSSKAATI KAFQEKGAIV IYGRVNNKEF MEMILKKYEI NVVISAIGGS
101 DGLLEQLTLV EAMKSINTIK RFLPSEFGHD VDRADPVEPG LTMYKQKRLV
151 RRVIEESGIP YTYICCNSIA SWPYYDNCHP SQLPPPLDQL HIYGHGDVKA
201 YFVDGYDIGK FTMKVIDDER TINKNVHFRP SNNCYSMNEL ASLWENKIAR
251 KIPRVIVSED DLLAIAAENC IPESVVAPIT HDIFINGCQV NFKIDGIHDV
301 EIGTLYPGES VRSLEECYEK FVVMAADKIH KEETGVTAGG GGTTAMVEPV
351 PITASC
```

FIGURE 31

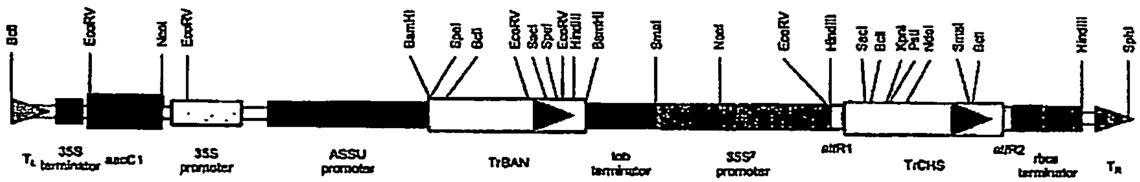


pPZP221:35S²TrLARc sense



pPZP221:35S²TrLARc anti

FIGURE 32



pPZP221:ASSU::BAN:35S²::CHS

FIGURE 33